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Preparation and characterization of a site-directed mutant of Escherichia coli L-threonine dehydrogenase

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PREPARATION AND CHARACTERIZATION OF A SITE-DIRECTED MUTANT OF *ESCHERICHIA COLI L*-THREONINE DEHYDROGENASE

by

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November 1999

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemistry

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ABSTRACT

PREPARATION AND CHARACTERIZATION OF A SITE-DIRECTED MUTANT OF *ESCHERICHIA COLI L*-THREONINE DEHYDROGENASE

By
Qian Zhou

Threonine dehydrogenase from *E.coli* is a member of the medium-chain, Zn^{2+}-containing alcohol dehydrogenase family. A site-directed mutant of theronine dehydrogenase was prepared at position 88 by replacing the native glutamate with cysteine using polymerase chain reaction. The mutated enzyme, designated TDH E88C, was expressed and purified to homogeneity in a three-step chromatographic procedure. The activity of the mutant after each step of purification was determined by a specific assay. Both the total activity and the specific activity of TDH E99C were significantly decreased compared to the wild-type TDH. The mutant enzyme was not sufficiently stable to obtain reliable V_{max} or K_m values, preventing a quantitative comparison to the wild type enzyme. Therefore, a definitive conclusion on the role of the Glu-88 remains unresolved, pending further studies in the future.

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LIST OF ABBREVIATIONS

ADH	Alcohol dehydrogenase
AKB	2-Amino-3-ketobutyrate
Amp	Ampicillin
CAT	Chloramphenicol acetyl transferase
Cd^{2+}	Cadmium (II) ion
Co^{2+}	Cobalt (II) ion
CoA	Coenzyme A
DEAE	Diethylaminoethyl
DNA	deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EXAFS	Extend X-ray absorption fine structure spectroscopy
GSH	Reduced glutathione
HPLC	high performance liquid chromatography
kbl	Gene coding for *E.coli* 2-amino-3-ketobutyrate: CoA lyase
LADH	Horse liver alcohol dehydrogenase isozyme E
IPTG	Isopropyl-1-thio-β-galactopyranoside
ITV	p-Iodonitrotetrazolium violet
λ_{max}	Wavelength of maximum absorptivity
LB	Luria-Bertani broth
LB-agar	Luria-Bertani agar

m/z	mass-to-charge ratio
NAD^+	β-Nicotinamide adenine dinucleotide, oxidized form
NADH	β-Nicotinamide adenine dinucleotide, reduced form
NADPH	β-Nicotinamide adenine dinucleotide phosphate, reduced form
dNTP	deoxynucleotide 5'-triphosphate
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PhMS	Phenazine methosulfate
PLP	Pyridoxal 5'-phosphate
R_m	Relative migration
RNase	Ribonuclease
SDH	Sorbitol dehydrogenase
SDS	Sodium dodecyl sulfate
SSB	(1S, 3R) 3-butylthiolane 1-oxide
TB	Terrific broth
tdh	Gene coding for *E.coli* TDH
TDH	Threonine dehydrogenase
TDH WT	Threonine dehydrogenase wild type
TE	Tris/EDTA buffer
TEMED	N,N,N,N-Tetramethylenediamine
X-gal	5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside
XDH	Xylitol dehydrogenase
YADH-1	Yeast (*Saccharomyces cerevisiae*) ADH isozyme I

LIST OF SYMBOLS FOR AMINO ACIDS

Amino acid	Three-letter abbreviation	One-letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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INTRODUCTION

L-threonine dehydrogenase catalyzes the NAD^+-dependent oxidation of threonine to aminoacetone and based on primary sequence homology, it is a member of the zinc-containing medium-chain alcohol dehydrogenase family.

❖ Alcohol dehydrogenase families

Alcohol dehydrogenases and related enzymes constitute a complex system of different enzymes, classes, isozymes, and allelic variants derived from gene duplications at minimally five different levels (Figure 1).

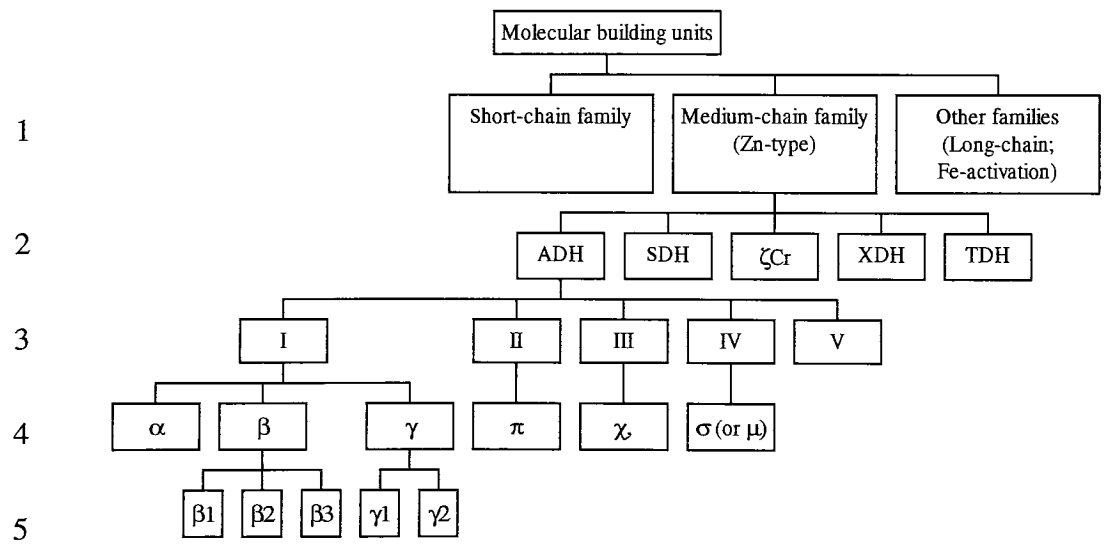

Figure 1. Schematic representation of alcohol dehydrogenase relationships with emphasis on medium chain alcohol dehydrogenase family.[1] Five different levels of relationship are distinguished, level 1 represents family multiplicity, level 2 represents enzyme multiplicity, level 3 represents class multiplicity, level 4 represents isoenzyme multiplicity. Level 5 represents allelic variants. ADH, alcohol dehydrogenase, SDH, sorbitol dehydrogenase; ζCr, zeta-crystallin; XDH, xylitol dehydrogenase; TDH, threonine dehydrogenase. I-V, ADH classes; α, β, γ, ADH class I subunits; π, ADH class II subunit; χ, ADH class III subunit; σ (or μ), ADH class IV subunit.

❖ **First level: family multiplicity**

The most distant relationships are the separate protein families with different primary amino acid sequences (especially highly conserved residues or conservatively replaced residues), catalytic mechanisms (such as metal ion requirement) and varied subunit sizes. The zinc-containing medium-chain alcohol dehydrogenase family is the one that has approximately 370 amino acid per subunit and typically requires zinc at the active site while the short-chain dehydrogenase family usually contain 250-300 amino acids per subunit functioning without metal cofactors.[2]

The zinc-containing medium-chain alcohol dehydrogenase family has been studied most extensively such as liver alcohol dehydrogenases, *E.coli* threonine dehydrogenase, liver sorbital dehydrogenase. The short-chain dehydrogenase family includes about 50 different enzymes known as mammalian sugar, steroid, prostaglandin (non-alcohol) dehydrogenases and insect alcohol dehydrogenases. Examples are *Drosophila* alcohol dehydrogenase, ribitol dehydrogenase from *Klebsiella aerogenes*[3] and glucose dehydrogenase from *Bacillus megaterium*.[4] No short-chain ethanol dehydrogenase has been identified in human tissues yet. Apart from the short-chain family and the medium-chain family, "other families" of alcohol dehydrogenase also exist and include a long-chain family and a family of iron-activated enzymes. They are known from prokaryotic and fungal sources such as an alcohol dehydrogenase from *Zymomonas mobilis*[5] and ADH_4 from the yeast *S. cerevisiae*,[6] but have yet not been studied in great detail.

The NAD^+ binding units are the segments of maximum similarity among many enzymes,[7] The short-chain dehydrogenases have the characteristic β-stranded NAD^+-binding fold[8] in the N-terminal region, rather than in the C-terminal region typical of the medium-chain family.

The enzymes in the medium-chain alcohol dehydrogenase family typically contain a catalytically active zinc atom at the active site, but it is not an absolute characteristic of the family. ζ-Crystallin, an enzyme initially discovered as a lens crystallin[9] and later found with NADPH: quinone oxidoreductase enzymatic activity,[10] exhibits a typical overall protein homology but lacks the zinc ligands and the zinc atom.[11] Some members of this family of alcohol dehydrogenases also has a second zinc atom per subunit ("structural" zinc); although it is not found in some family members, including sorbitol dehydrogenase.[12]

❖ **Second level: enzyme multiplicity**

The second level of gene duplications represents the different enzymes based on substrate specificity. They are distantly related and with no substrates in common, hence constituting truly different enzymes, however enzymes at this level are related conformationally. Thus, model building is possible and relationships regarding zinc-binding properties, subunit interactions and active site structures can be deduced from comparisons.[13]

❖ **Level three: class multiplicity**

Level three gives rise to different classes of enzymes in ADH. They do not cross-hybridize which means the subunit of each different class can't combine freely to form heterodimers, only class-specific homodimers can be formed. They are different in expression, regulation or substrate specificity. Nevertheless, the different classes have some activity toward a common substrate, ethanol.

At least five classes of alcohol dehydrogenase are found in tissues of humans and many other vertebrates. Class I represents the classical liver enzyme, exhibiting considerable activity toward ethanol, sensitivity to pyrazole inhibition, and frequent existence of isozymes. Class III is a dual enzyme that has alcohol dehydrogenase activity and also is identical to the glutathione-

dependent formaldehyde dehydrogenase.[14] This enzyme is the form of most distant origin and involved in basic cellular defense mechanisms.[15] Class IV is present in stomach[16] and has the highest ethanol activity of all classes thus far characterized.[17] Class II and classes V have not been analyzed thoroughly.

❖ **Fourth level: isozyme multiplicity**

The fourth level of gene duplication during mammalian evolution constitutes isozyme formation. This level was discovered quite recently and has been characterized only for the mammalian class I enzymes. In humans, three isozyme subunits, α, β and γ, associate freely into all possible dimeric combinations.[18]

❖ **Fifth level: allelic variants**

In addition to these four levels, further multiplicity derives from allelic variability. At least three alleles (β_1, β_2, β_3) have been characterized for the human class I β form and two (γ_1, γ_2) for the class I γ form.[19] These isozymes are responsible for the main ethanol metabolism in liver but exhibit differences in substrate specificity and catalytic efficiency. Steroid dehydrogenase activity has only been shown for isozymes containing the γ subunit[20] and they are the only ones that can be inhibited by testosterone.[21]

❖ **Role of Zn^{2+} in enzyme structure and activity**

Zinc is an important component to many enzymes. It has been found to be an integral component of nearly 300 enzymes in different species. About a decade ago, the role of zinc in gene expression began to attract interest.[22] Zinc also stabilizes the structure of proteins and nucleic acids, plays an important role in catalysis, preserves the integrity of subcellular organelles, participates in transport processes and viral immune phenomena.[23] Its nutritional

essentiality has focused attention on the pathology and clinical consequences of both its deficiency and toxicity.[24]

Members of the medium chain zinc-containing alcohol dehydrogenase family contain catalytic zinc atoms and, in some cases, structural zinc atoms. The active site zinc in ADH is bound to three amino acid side chains: The first two ligands, cysteine and histidine, are conserved in the ADH active site. The third ligand may be either cys, glu, or asp (Table I). The forth ligand is a water molecules if inhibitors or substrate are not present.

The crystal structure of horse liver alcohol dehydrogenase (EC 1.1.1.1) is already known and hence can serve as a model for catalytic and structural zinc sites. It is a dimeric molecule, each subunit is made up of two domains, the coenzyme binding domain, which contains residues for NAD binding, and the catalytic domain which contains most residues responsible for substrate (ethanol) binding including an active site zinc ion (Fig. 2).

The structural zinc atoms are coordinated to four cysteine residues, which are highly conserved among many enzymes. The structural zinc is close to the surface of the molecule and forms tetrametric geometry with its cysteine ligands (Fig. 2 and Fig. 3). This structural zinc atom might primarily affect local structure and conformation.[25]

The ligands of the active-site zinc atom of horse liver alcohol dehydrogenase were identified by x-ray crystallography. The catalytic zinc is located in a deep cleft between the catalytic and coenzyme-binding domains. It is bound to residues Cys-46, His-67 and Cys-174 (Fig. 4 and Fig. 5). The involvement of both zinc and NADH in the catalytic process requires suitable alignment of amino acid residues that can provide for both metal chelation and coenzyme binding sites. In LADH this has been accomplished by the use of residues Cys-46 and Arg-47 as zinc and NADH-binding ligands, respectively, with two cysteines and one histidine as

ligands to the active-site zinc. When NAD binds to the enzyme, the catalytic domains rotate 10^{o} towards the coenzyme binding domain, tightening the interactions with NAD and excluding water from the central part of the active site.[26]

The reaction catalyzed by LADH is sequentially ordered:[27] NAD^{+} binds to the enzyme first. Then the alcohol substrate displaces the fourth ligand of the catalytic Zn^{2+}-a water molecule. The hydroxyl group of the substrate ligates to this metal ion. Binding to the positively charged NAD^{+} and the zinc decreases the pK_a of the alcohol hydroxyl group, triggering a rapid deprotonation of the alcohol. Hydride transfer from the substrate to NAD^{+} results in an aldehyde product. This is followed by dissociation of the aldehyde product and a subsequent association of a water molecule to the catalytic metal ion and then dissociation of NADH.[28]

Protein	Residue number		
	46	67	174
Horse E	T G I C R S D	I A G H E A A	L I G C G F S
Human α	V G I C G T D	I L G H E A A	L I G C G F S
Human β	V G I C R T D	I L G H E A A	L I G C G F S
Human γ	A G I C R S D	I L G H E A A	L I G C G F S
Human π	T S L C H T D	I V G H E A A	L I G C G F S
Human χ	T A V C R S D	I L G H E G A	L I G C G I S
E coli	T A I C G T D	V V G H E Y V	I F - **D** P F G
Yeast 1	S G V C H T D	V G G H E G A	P V L C A G I
Yeast 2	S G V C H T D	V G G H E G A	P I L C A G I
Yeast 3	S G V C H T D	V G G H E G A	P I L C A G V
Yeast 4	T G V C H T D	I G G H E G A	P I M C A G I
Sheep SDH	V G I C G S D	V L G H E A S	L I **E** P L S

Table I. Active-site zinc ligands for alcohol dehydrogenases. *E.coli* refer to *E.coli* TDH. Yeasts 1 and 2 are respectively *Saccharomyces cerevisiae* cytosolic isozymes 1 and 2, yeast 3 is a *S. cerevisiae* mitochondrial enzyme, and yeast 4 is a *Schizosaccharomyces pombe* enzyme. Refer to Fig. 1. for human α-χ.

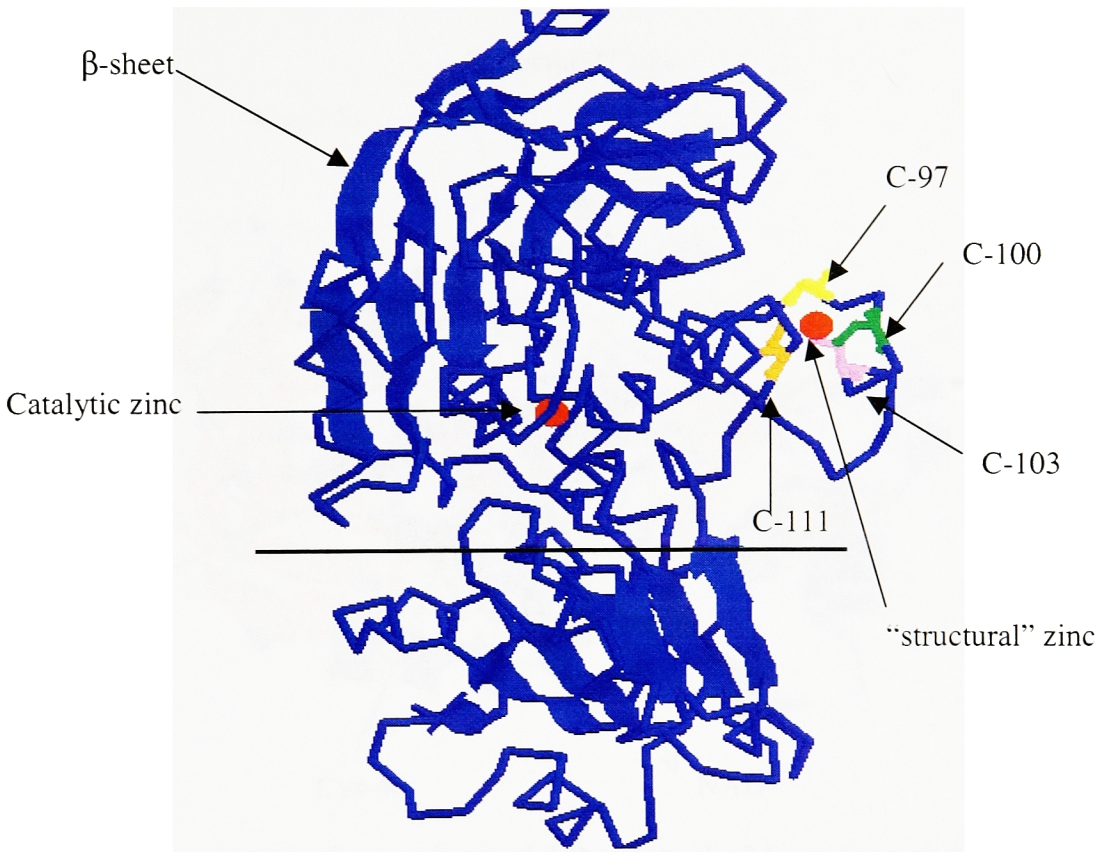

Figure 2 Representation for one subunit of horse LADH (protein data bank file 1BTO) showing the structural Zn^{2+} and its ligands. The domain above the horizontal line is the catalytic domain and below is the co-enzyme binding domain.[29]

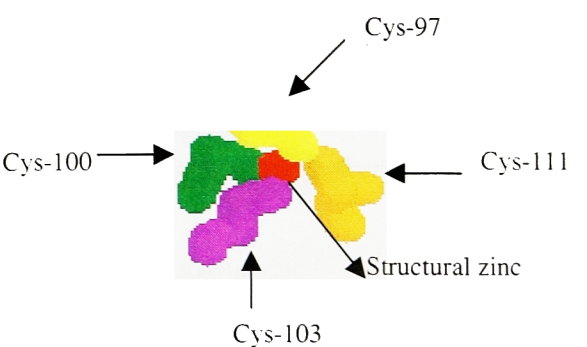

Figure 3. A close up view of structural zinc and its cysteine ligands.

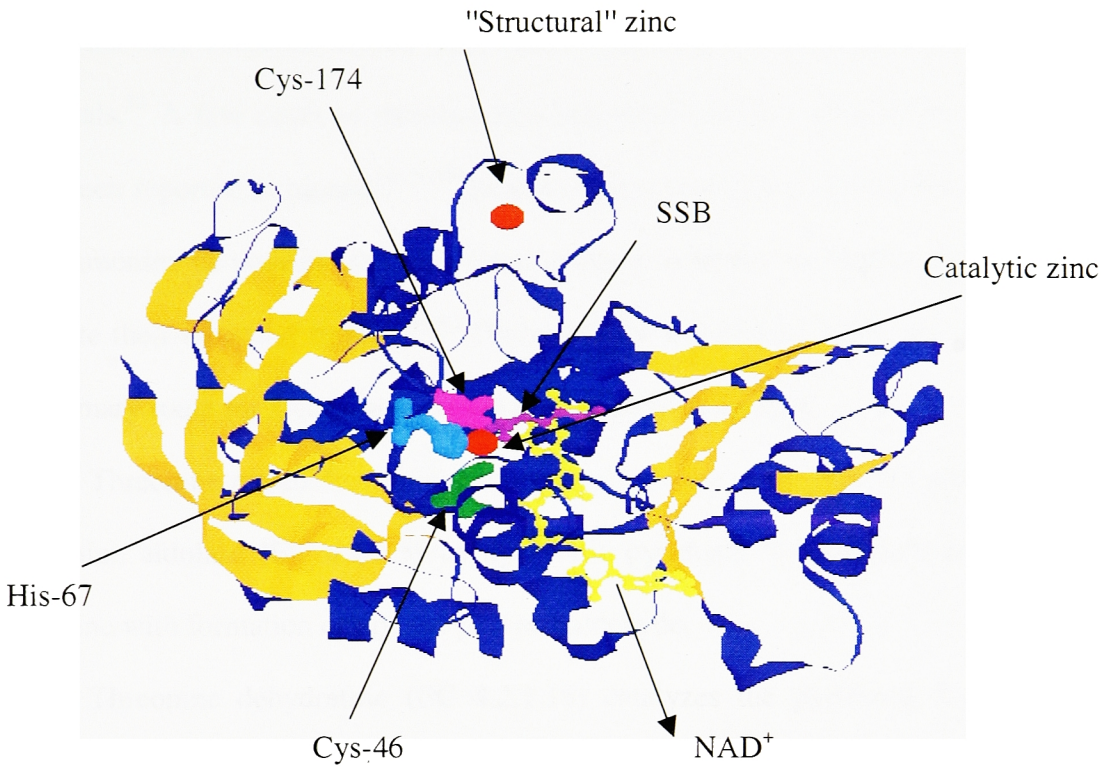

Figure 4. Representation of one subunit of 3-D structural of horse liver alcohlol dehydrogenase complexed with co-enzyme NAD^+ and substrate SSB (Protein data back file 1BTO) showing the catalytic zinc and its ligands. SSB stands for (1S, 3R) 3-butylthiolane 1-oxide.

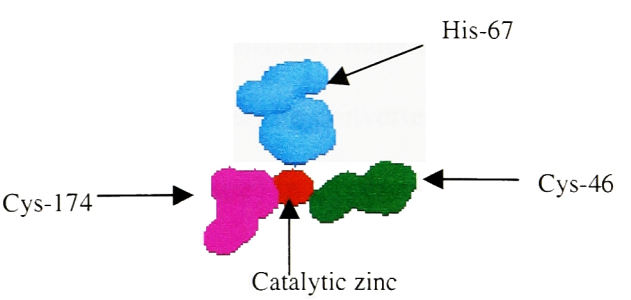

Figure 5. A close-up look of active site Zn^{2+} and its ligands.

L-threonine is the 2(S), 3(R) stereoisomer of 2-amino-3-hydroxybutyrate. It was isolated from hydrolysates of the blood protein, fibrin, and determined by W. C. Rose and his colleagues to be the last essential amino acid required for the normal growth and development of mammals.[30] A few cases of threoninemia that might involve defects in threonine metabolism have been reported in human.[31,32,33] Recent clinical trials have demonstrated that administration of *L*-threonine to patients suffering from multiple sclerosis or familial spastic paraparesis can alleviate their muscular rigidity.[34,35] Therefore, the research of TDH, the enzyme thought to be the primary route for *L*-threonine metabolism is of great interest.

Threonine metabolism follows three main routes in prokaryotes and eukaryotes (Fig. 6). Threonine aldolase (EC 4.1.2.5) catalyzes the pyridoxal 5'-phosphate-dependent cleavage of threonine with formation of glycine plus acetaldehyde.

Threonine dehydratase (EC 4.2.1.16) catalyzes the pyridoxal 5'-phosphate-dependent deamination of *L*-threonine with production of 2-ketobutyrate (Fig. 6).

Threonine dehydrogenase (EC 1.1.1.103) is the most important enzyme involved in *L*-threonine metabolism (Fig. 6). It catalyzes the NAD^+-dependent oxidation of threonine to form an unstable intermediate, 2-amino-3-ketobutyrate, which either decarboxylates to form aminoacetone which can be sterospecifically reduced to *D*-1-amino-2-propanol and incorporated in vitamin B_{12} biosynthesis,[36] or it can be converted to acetyl CoA plus glycine by the enzyme aminoketobutyrate ligase (AKB ligase) in a coenzyme A dependent reaction.[37] Successively the glycine may participate in serine biosynthesis in *E.coli* under certain environmental conditions.[38] TDH, therefore, initiates the conversion of *L*-threonine into many metabolic intermediates and plays a major role in threonine metabolic pathways.

Threonine dehydrogenase activity is widespread and has been detected in cell-free extracts of such microorganisms as *E.coli*,[39] *Staphylococcus aureus*,[40] *Corynebacterium* sp. B6,[41] *Clostridium stricklandii*,[42] and *Bacillus subtilis*[43] as well as in liver homogenates of the rat,[44] guinea pig,[45] bullfrog,[46] pig,[47] chicken,[48] and goat.[49] TDH has been widely accepted as playing the major role in initiating the metabolism of threonine in both prokaryotes and eukaryotes, while the contribution of threonine aldolase or dehydratase to the degradation of *L*-threonine is relatively small.

TDH has been isolated in homogeneous form from six sources: *E.coli*, pig liver, *C.stricklandii*, *S. marcescens*, goat and chicken liver. Some characteristics of these enzymes are listed in table II. TDH from goat[49] and chicken liver[48] are each composed of only a single polypeptide while that from *C.stricklandii*[42] and *S. marcescens*[50] is dimeric. Those from pig liver[47] and *E.coli*[39] are similar to each other in that they are both homotetrameric proteins. *E.coli* TDH was the first to be purified to homogeneity. It was isolated from extracts of a constitutive mutant strain of *E.coli* K-12, which designated as SBD76. This mutant had elevated levels of the enzyme as compared to wild type strain when grown on *L*-threonine as the sole carbon source.[51]

E.coli threonine dehydrogenase has been extensively characterized. It is composed of four identical subunits (M_r= 148,000), each having 341 amino and a molecular weight of 35 kDa. The purified *E.coli* K-12 enzyme showed K_m values for *L*-threonine and NAD^+ of 1.43 mM and 0.11 mM (in 50 mM Tris-HCl buffer pH 8.4 and 37°C), respectively, while the V_{max} was 34 U/mg and the enzyme had a pH optimum of 10.3. Added Cd^{2+} or Mn^{2+} increases the V_{max} 9- or 24-fold, respectively.[52] It only contains 1 Zn^{2+} atom/subunit.[53] The substrate specificity of the purified enzyme is broad: *L*-threonine, *L*-threonine amide, and *D,L*-2-amino-3-hydroxypentanoic acid; *D*-threonine, however, is inactive.[51]

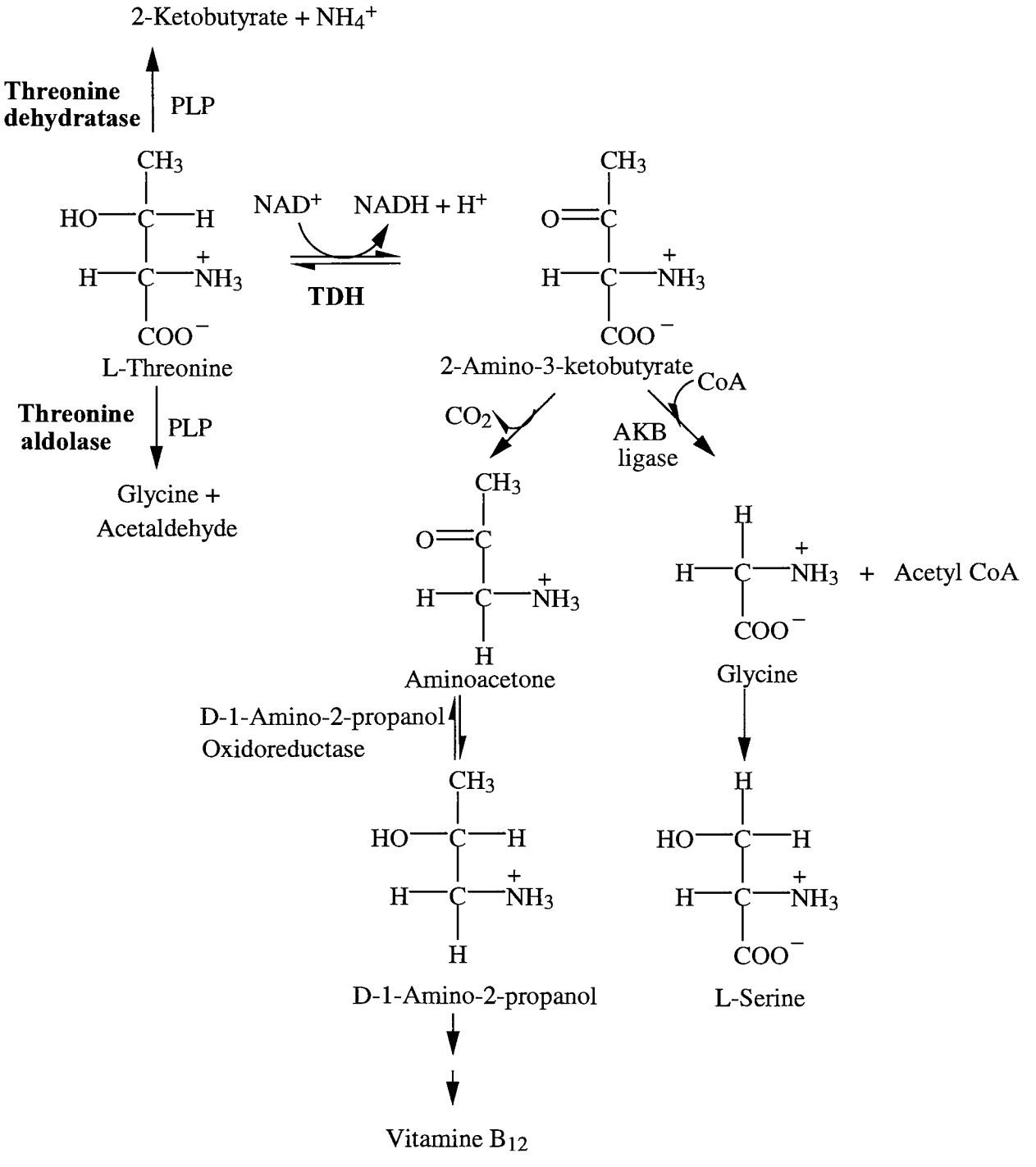

Figure 6. Metabolic pathways for *L*-threonine. PLP stands for pyridoxal 5'-phosphate. AKB ligase stands for 2-amino-3-ketobutyrate ligase.

Table II. Characteristics of homogeneous threonine dehydrogenases.

Source	Subunit M_r (kDa)	# of Subunits	pH Optimun	Specific Activity (U/mg)	K_m for NAD^+ (mM)	K_m for Threonine (mM)
E.coli	37.2	4	10.3	34	0.11	1.43
Pig liver	25	4	8.4	30	0.1	5
C. stricklandii	33	2	9.0	234	0.1	18
Goat liver	89	1	8.5-8.6	8.45	1.0	5.5
Chicken liver	88	1	8.6-8.7	19	0.98	8.4

When *E.coli* tdh was cloned and sequenced, the deduced amino acid sequence showed that TDH was homologous with members of the medium-chain, Zn^{2+}-containing alcohol dehydrogenase family.[54] The amino acid sequence of *E.coli* TDH is most similar to sheep liver sorbitol dehydrogenase (SDH) and yeast alcohol dehydrogenase (28 and 27% identity, respectively), while it has 25% identity with that of horse liver alcohol dehydrogenase (LADH) and maize ADH_2.[55] Fig. 7. presents an alignment of the primary amino acid sequence of these enzymes with that of *E.coli* TDH.

The ligands to the active site zinc of LADH are Cys-46, His-67 and Cys-174. Based on computer modeling, it has been suggested that SDH also has a Cys-46, His-67 as ligands to the catalytic zinc but differs from the LADH in having a glutamate, Glu-153 which aligns with Cys-174 in LADH (Fig. 7), as the third ligand; glu-153 in SDH aligns with Asp-148 from *E.coli* TDH. Studies on the structures of horse liver alcohol dehydrogenase and its complexes with NAD^+ and substrate show two carboxyl groups near, but not directly ligated to, the active site zinc[29]. The carboxylate of Asp-49 forms a hydrogen bond to the imidazole of His-67, one of the zinc ligands, whereas Glu-68 is located behind the metal ion, opposite the substrate binding site. These two residues are conserved in many mammalian alcohol dehydrogenases,[56] higher plants[57] and fungi.[58] Glu-68 is conserved in *E.coli* TDH as well (which aligns to Glu-64 in *E.coli* TDH). Such comparison suggests that the residues containing carboxyl group might be involved as active zinc-ligands.

E.coli TDH is a homotetrameric protein which contains only one Zn^{2+} ion per subunit and the extend X-ray absorption fine structure (EXAFS) spectroscopic analyses suggests that it might be a non-catalytic zinc which probably is bound by cysteine residues 93, 96, 99, and 107,[59] comparable to the structural Zn^{2+} of horse liver ADH.

A few residues in *E.coli* have shown great importance in structure/function relationships. Methylation of His-90 (which aligns with Thr-94 in horse LADH[55]) with methyl *p*-nitrobenzenesulfonate[60] and site directed mutagenesis of this residue both suggested that His-90 may play a role in substrate recognition.[28] TDH activity was abolished by *p*-mercuribenzoate[51] and iodoacetate,[61] the residue modified was determined to be Cys-38[53] and site directed mutagenesis confirmed that Cys-38 as a probable ligand to the zinc.

The goal of the research presented in this thesis is to investigate the role of Glu-88 in reaction catalyzed by TDH by constructing a site-directed mutant enzyme, TDH E88C (Glu-88 to Cys-88). The thesis focuses on generating the mutant form of the enzyme, then purifying it to homogeneity using several chromatographic methods.

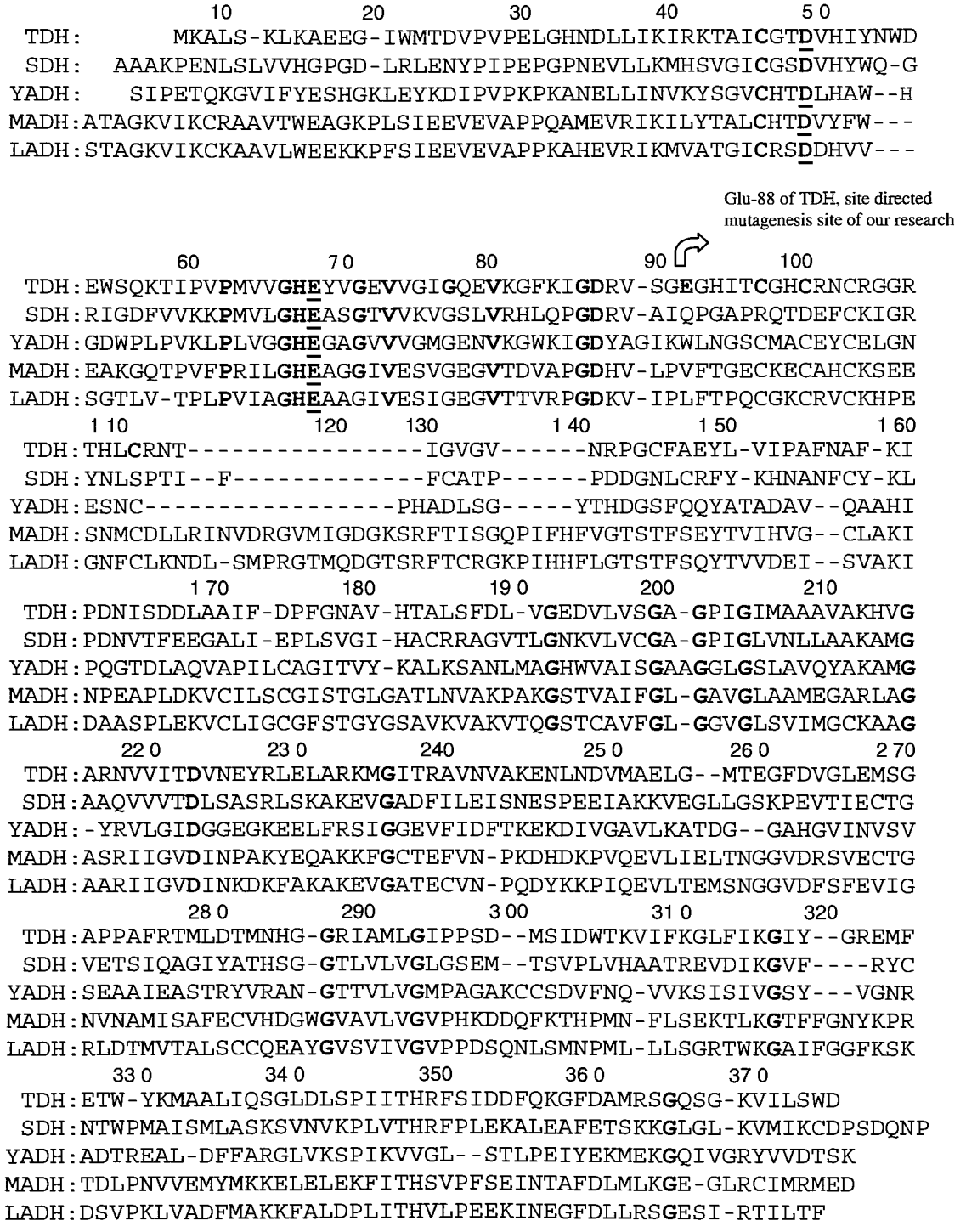

Figure 7. Amino acid sequence comparison of several alcohol dehydrogenases. *E.coli* threonine dehydrogenase (TDH); SDH (sheep liver sorbitol dehydrogenase); YADH (yeast alcohol dehydrogenase, isozyme 1); LADH (horse liver alcohol dehydrogenase, isozyme E). The numbers correspond to the LADH sequence. Residue numbers are for the amino acid corresponding to the last digit of the number[28]. The bold letters are conserved sequences and the bold underlined amino acids show the conserved carboxyl groups that might be involved in the active zinc binding site.

MATERIALS

Materials used for culturing *E.coli* SP1192 pAJWT cells were purchased from the sources listed: *E.coli* sp1192 pAJWT stock was a generous gift of Professor Eugene E. Dekker from the University of Michigan Dept. of Biological Chemistry; bacto-yeast extract from Becton Dickinson; bacto-tryptone from DIFCO Laboratories; glycerol from J.T.Baker; and ampicillin from Sigma.

General chemicals used routinely were reagent-grade products of the companies indicated: potassium dihydrogen phosphate, potassium hydrogen phosphate, sodium hydroxide, potassium chloride, hydrochloric acid, methanol, acetic acid, calcium chloride and sodium azide from J.T.Baker; Trizma hydrochloride, Trizma base and 2-mercaptoethanol from Sigma; ethanol from AAPER Alcohol and Chemical CO.

The BIGGER prep™ Plasmid DNA Preparation Kit was purchased from 5 Prime→ 3 Prime, Inc. The components included: Solution I (buffered RNase A solution), solution II (alkaline lysis solution), solution III (potassium acetate solution), DNA binding matrix suspension in guanidine-HCl, buffered salt solution, TE (pH 8.0) and sodium chloride.

The QuikChange™ Site-Directed Mutagenesis Kit was purchased from Stratagene, materials provided: *Pfu* DNA polymerase, 10x reaction buffer, *Dpn* restriction enzyme, oligonucleotide control primer #1, oligonucleotide control primer #2, pWhitescript™ 4.5-kb control plasmid, dNTP mix, Epicurian Coli® XL-Blue supercompetent cells and pUC18 control plasmid. Additional materials required: X-gal and IPTG from Life Technologies. All the oligonucleotide primers were synthesized at the University of Rochester Core Nucleic Acid Lab.

ABI PRISM™ dye terminator pre-mix DNA sequencing reagents were from Perkin-Elmer Corporation.

QIAprep Spin Miniprep Kit was ordered from QIAGEN. Reagents provided: RNase A, buffer P_1, buffer P_2, buffer N_3, buffer PB and buffer PE.

The materials used in the purification of threonine dehydrogenase were purchased from the companies noted: Poros 50 DEAE weak anion exchange packing and HQ high capacity strong anion-exchange column from PerSeptive Biosystems; Cibracron Blue F3GA from Sigma; and TOYOPEARL® HW-55S from Superlo, Inc.

Chemicals for agarose gel electrophoresis and polyacrylamide gel electrophoresis were purchased from the following resources: standard low electroendosmosis agarose, ammonium persulfate, SDS, sodium acetate, tris-acetate and EDTA from J.T.Baker; 30% acrylamide/Bis solution, TEMED, glycine, Coomassie blue stain, SDS-PAGE low range molecular weight standards, ethidium bromide and bromophenol blue from Bio-Rad Laboratories; 1kb DNA ladder from New England Biolabs, Inc, and DNA loading dye from Carolina Biologicals, Inc.

Reagents for protein and activity assays were purchased from the following companies: protein assay dye reagent concentrate from Bio-Rad Laboratories; bovine gamma-globulin from Worthington Biochemical Corporation; threonine from J.T.Baker; NAD^+, phenazine methosulfate and p-iodotetrazolium violet from Sigma.

Instrumentation used for experiments were from the sources listed: SORVALL® RC 5C Plus centrifuge and SLA-1500 and SA-600 rotors were from Dupont; Centra-CL2 centrifuge from International Equipment Company; desktop Biofuge 13 from Heraeus Instruments; Sonifier® sonicator from Branson Instruments, Inc; GeneAmp PCR System 2400 from Perkin Elmer; Waters™ 650 protein purification system and 486 tunable absorbance detector from

Waters; fraction collector (model FC 205) from Gilson; 37°C water bath, 37°C incubator, -85°C freezer from VWR Scientific; UV-VIS spectrophotometer model 8543 from Hewlett Packard; microplate reader from DYNEX Technologies; electrophoresis assemblies and electrophoresis power supply (model PAC 300) from Bio-Rad.

EXPERIMENTAL METHODS

1. Growth of *E.coli* cells containing pAJWT plasmid

E.coli SP1192 is a tdh$^-$ strain in which the gene coding for chloramphenicol acetyl transferase (CAT) has been inserted into *tdh* gene.[62] The TDH gene has been cloned into a plasmid called pDR121[38] and then genetically subcloned to the plasmid pAJ (sometimes called pTDH). This plasmid also contains the gene for ampicillin resistance that is an important feature for bacterial selection when grown on media containing ampicillin. The plasmid containing the wild type tdh gene is called pAJWT.

E.coli SP1192 pAJWT stock cells were grown in LB containing 60 μg/mL ampicillin, diluted with an equal volume of sterile glycerol and stored at –82°C. One aliquot was thawed and maintained at –20°C for use. Luria Broth (LB) was prepared as indicated:[63] 10 g NaCl, 5 g bacto-yeast extract, 10 g bacto-trptone were dissolved in one liter of deionized water and sterilized by autoclaving. LB-ampicillin agar plates were prepared as follow: the liquid LB medium was prepared according to the recipe given above, but before autoclaving, 15g/liter bacto-agar were added. After autoclaving, broth and plate media were allowed to cool to 50° before adding 60μg/mL of ampicillin. Finally the medium was poured to plates when it was cool. Terrific Broth (TB) was prepared as follows:[63] 12 g bacto-tryptone, 24 g bacto-yeast extract, and 4mL glycerol were dissolved in 900 mL deionized water and autoclaved. 2.31 g KH_2PO_4 and 12.54 g K_2HPO_4 were dissolved in 100 mL ddH_2O and autoclaved in a separate flask. The two solutions were mixed after cooling to 60° or less.

A 50 mg/mL ampicillin solution was prepared and sterile filtered (0.22 μm sterile filter disk) and stored at 4°C. Ampicillin (6 μl of 50 mg/mL stock) was added to 5 mL of LB media; 5 μl of *E.coli* SP1192 pAJWT stock were then inoculated into the same media. The cells were incubated in a 37°C shaker for 16 hours. Ampicillin (0.6 mL of 50 mg/mL stock) was added to 500 mL TB that was inoculated with 5 mL of *E.coli* SP1192 pAJWT culture. The cells were incubated in a 37°C shaker overnight with vigorous shaking and harvested by centrifugation at 10,000 x g for 10 min using RC-5C Plus centrifuge equipped with a SLA 1500 rotor. Supernatant was decanted and the cell paste was transferred to a 50 mL conical centrifuge tube and stored at –20°C in a non-defrosting freezer. The wet weight of the cells was recorded.

2. Site-directed mutagenesis of threonine dehydrogenase in *E.coli* using PCR

2.1. Plasmid DNA preparation

The BIGGERprep™ Plasmid DNA Preparation Kit was used to isolate the plasmid pAJWT. The cell pellet from 5 mL of cells grown overnight in LB containing 60 μg/mL ampicillin was completely suspended in 15 mL of supplied Solution I (buffered RNase A solution), then 23 mL Solution II (alkaline lysis solution) was added and mixed well until the lysate looked clear with no visible clumps. The bacterial lysate was neutralized by adding 11 mL Solution III (potassium acetate solution) and mixed by gentle inversion until white material was broken into small clumps. Then the mixture was centrifuged at 23,000 x g for 5 min and the supernatant was carefully transferred to a fresh centrifuge bottle. 40 mL of well-mixed BIGGERprep DNA Binding Matrix Suspension was added to the recovered supernatant above to efficiently bind the plasmid DNA. The mixture in the centrifuge bottle was centrifuged at 23,000 x g for 5 min and the supernatant was decanted. Diluted Purification Solution (10 mL) was added to the pellet and mixed well by vigorous shaking (75 mL of Purification Solution Concentrate

was diluted with 75 mL of 95% ethanol). The mixture was poured into a BIGGERprep Spin Column that had been inserted into a 50 mL conical centrifuge tube. Another 10 mL diluted Purification Solution was added to the centrifuge bottle to wash the residual matrix from bottle sides. The wash was poured into the same BIGGERprep Spin Column. The Spin Column/centrifuge tube assembly was centrifuged at 2,000 x g for 5 min. Then 10 mL more of the diluted Purification Solution was added to the same BIGGERprep Spin Column to further purify the bound plasmid DNA and centrifuged at 2,000 x g for 5 min. Then the BIGGERprep Spin Column was transferred to a fresh, sterile, 50 mL conical centrifuge tube. The purified plasmid DNA was eluted by 3 mL TE, pH 8.0 and centrifuged at 2,000 x g for 5 min. The eluted purified plasmid DNA was carefully transferred to the provided "precipitation" tube and 70 μL 5 M NaCl were added followed by 6 mL 95% ethanol. The mixture was centrifuged at 16,000 x g for 5 min. The pellet was washed with 70% ethanol twice and air-dried. After the pellet was dry, the pellet was resuspended in 1 mL nanopure water and stored at 4°C. DNA agarose gel electrophoresis was carried out to evaluate the extraction.

The concentration of the isolated plasmid was determined by UV-VIS spectrophotometer. The plasmid was diluted 100-fold in ddH_2O. The spectrum was taken and the absorbance values at 280 and 260 nm were recorded. The concentration of the plasmid DNA was estimated using A_{260}=1.0 for 50 μg/mL of plasmid DNA solution. The ratio of A_{260}/A_{280} was used to evaluate the presence of protein in the plasmid preparation; a ratio ≥ 1.8 indicated acceptable removal of proteins.

2.2 Preparation of oligonucleotide primers

Two primers were prepared by the University of Rochester Core Nucleic Acid Lab using Nucleic Acid Synthesis System ABI 392 and ABI Masterpiece™ reagents from Perkin Elmer

Corporation. They were complementary to opposite strands of the double-stranded plasmid DNA template and contained the changes of the codon for residue 88 of TDH from Glutamate (5'-GAA) to Cysteine (5'-TGC, see Figure 9). The primer sequence (mutation points are underlined) that would extend downstream during thermal cycling was 5'-CGTTTCTGGCTGCGGCCATATCA-3'; the primer sequence that would extended upstream was 5'-TGATATGGCCGCAGCCAGAAACG-3'.

The site-directed mutagenesis PCR protocols below were from Stratagene QuikChange™ Site-Directed Mutagenesis Kit.

2.3. Setting up the mutagenesis reactions for PCR

A control reaction was prepared as indicated below: 5 μL of 10× reaction buffer, 2 μL of pWhitescript™ 4.5-kb control plasmid (5 ng/μL), 1.25 μL of oligonucleotide control primer #1 (100 ng/μL), 1.25 μL of oligonucleotide control primer #2 (100 ng/μL), 1 μL of dNTP mix and double-distilled water (ddH_2O) to a final volume of 50 μL. Reaction buffer (10×) consisted of 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl (pH 8.8), 20 mM $MgSO_4$, 1% Triton® X-100 and 1 mg/mL nuclease-free bovine serum albumin. The sample reaction contained the following components: 5 μL of 10× reaction buffer, varying concentrations of pAJWT DNA template (5 ng, 10 ng, 100 ng) for each reaction tube, 125 ng of the two oligonucleotide primers, 1 μL of dNTP mix to a final volume of 50 μL. *Pfu* DNA polymerase (2.5 U) was added to both of the reaction mixtures.

2.4 Cycling the reaction

The following program was cycled 16 times and kept at 4°C until removal.

Segment	Cycles	Temperature	Time	
1	1	95°C	30 seconds	
2	16	95°C	30 seconds	Denaturation
		55°C	1 minute	Annealing
		68°C	10 minutes	Elongation

2.5. Digesting the products

Following the PCR reaction, *Dpn* I restriction enzyme (10 U) was added to each amplification reaction tube and mixed thoroughly. The reaction mixtures were then centrifuged for 1 minute and immediately incubated at 37°C for 1 hour to digest the parental double stranded DNA. The *Dpn* I endonuclease can recognize and digest the C-6 methylated guanine in the target sequence 5'-G_{m6}ATC-3, so the parental DNA template is cut into pieces and leaves the mutation-containing synthesized DNA intact.[64]

2.6. Transforming into Epicurian coli SL1-Blue supercompetent cells

One μL *Dpn* I-treated DNA from the control and sample reactions was added to separate 50 μL aliquots of the supercompetent cells provided. The reaction tubes were incubated on ice for 30 minutes. Then the reactions were heat shocked for 45 seconds at 42°C, followed by placing them on ice for 2 min. 0.5 mL of LB broth preheated to 42°C was added to each reaction tube and incubated at 37°C for 1 hour with shaking. 250 μL of the control transformation reaction was plated on LB-ampicillin agar plate that had been spread with 20 μL of 10% (w/v) X-Gal and 20 μL of 10 mM IPTG. The entire volume of the sample reaction was plated on LB-ampicillin agar plate. All the plates were incubated at 37°C overnight.

3. Agarose gel electrophoresis of DNA

Agarose (0.8%) was prepared in TAE buffer (40 mM tris-acetate, 20 mM sodium acetate, 1mM EDTA, pH 8.0). A 1kb ladder was used as standard. DNA samples at various stages of the experiments were analyzed. The gel was run at 100 V for about 1 hour until the tracking dye approached the end of the gel. Then the gel was stained in 0.2 μg/mL ethidium bromide for 15 min and destained with distilled water. A picture of the gel was taken on the UV light box by Kodak digital camera (Model DC40) connected to a ZEOS 486 computer running Windows NT 4.0. The picture was analyzed and printed using Kodak Digital Science 1D software (Figure 10).

4. Growth of Epicurian Coli SL1-Blue cells containing PCR mutated plasmid

Randomly twenty colonies from the LB-ampicillin plate containing the sample reaction which started with 10 ng DNA template concentration in the PCR reaction mix were selected and sterilely inoculated in separate tubes containing 5 mL LB media with 60 μg/mL ampicillin. The cells were cultured overnight on the 37°C shaker. Cells (0.5 mL from each of 5 mL culture tubes) were diluted in equal volume of sterile glycerol and frozen in liquid nitrogen and then kept in -85°C freezer for long term storage.

5. Isolation of PCR mutated plasmid from Epicurian Coli SL1-Blue cells

The QIAprep Plasmid Preparation Kit was used for plasmid isolation. All centrifugation steps for the miniprep were performed on Heraeus microfuge at maximal speed (10,000 x g or 13,000 rpm). Randomly six of the twenty 4.5 mL overnight cultures from procedure 4 were selected for further experiments. They were centrifuged at 13,000 rpm in a centrifuge for 5 min. Kit reagents were prepared according to the manufacturers specifications: the supplied RNase A was diluted in 2 mL ddH_2O and then added to Buffer P1 and stored at 4°C; an equal amount of 95% ethanol was added to Buffer PE.

The cell pellets were resuspended in 250 μL Buffer P1 and transferred to microfuge tubes. Buffer P2 (250 μL) was added to each tube and gently inverted 4-6 times to mix. Then 350 μL of Buffer N3 was added and the tubes were inverted immediately but gently 4-6 times until the solution became cloudy and very viscous. All the tubes were centrifuged for 10 min and a compact white pellet formed. The QIAprep spin columns were placed in 2 mL collection tubes and the supernatants were applied to the QIAprep column assemblies. They were centrifuged for 60 sec and the filtrates were discarded. QIAprep spin columns were washed by 0.5 mL Buffer PB and centrifuged for 60 sec. 0.75 mL of Buffer PE was added to each QIAprep spin column and centrifuged for 60 sec. QIAprep spin columns were centrifuged for an additional 1 min to remove residual wash buffer. Then QIAprep spin columns were placed in clean 1.5 mL microfuge tubes and 50 μL TE buffer was added, the assemblies were centrifuged for another 1 min after standing for 1 min. The filtrates containing the plasmids were collected in separate microfuge tubes.

6. Cycle sequencing to evaluate the tdh gene of the PCR mutated plasmid

The protocol was taken from the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit.[65] The four sequencing primers were synthesized at the University of Rochester Core Nucleic Acid Laboratory. The positions where they will anneal to the tdh template are shown on Figure 8. The sequence of two external primers were: 031, 5'-ACGCGTGCAGTAGAAGCAATTTACGCG-3'; 030, 5'–CGCGTGCAGTAGAAGC ATTTACGCG-3'. The sequence of two internal primers were: 636, 5'-GTCGTGGGCCATGAATATGT-3'; 103, 5'-CGATAGACATATCAGACGGC-3'.

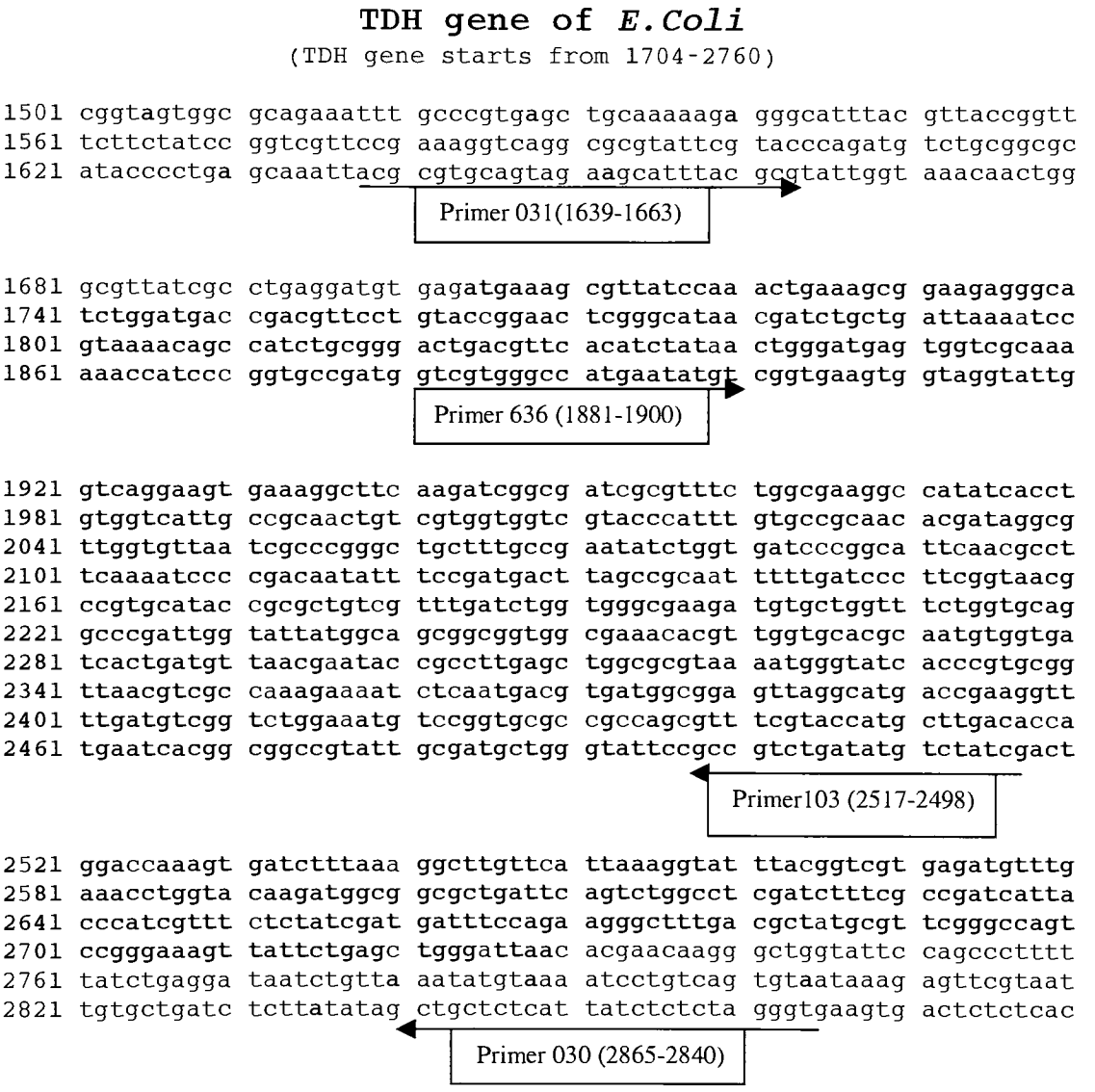

Figure 8. The complete sequence of the tdh gene from *E.coli* (bold) and positions of four sequencing primers (above arrows).

Each sequencing reaction consisted of 8.0 μL Terminator Ready Reaction Mix (double-stranded plasmid DNA 0.4 μg, primer 3.2 pmole and ddH_2O to a total volume of 20 μL). It was cycled on the GeneAmp PCR system 2400. The thermal cycling was begun as follows:

- Rapid thermal ramp to 96°C
- 96°C for 10 sec
- Rapid thermal ramp to 55°C
- 55°C for 5 sec
- Rapid thermal ramp to 62°C
- 62°C for 4 min

The thermal cycle was repeated for 25 cycles and kept at 4°C until removal. The reaction tubes were sent to University of Rochester Core Nucleic Acid Laboratory for DNA sequencing.

7. Expression of the mutated gene of threonine dehydrogenase in *E.coli*

These protocols were taken from *Molecular Cloning: A Laboratory Manual*.[63]

7.1. Competent *E.coli* SP1192 cells preparation

An overnight culture of *E.coli* SP1192 cells (1 mL) was aseptically transferred to 100 mL LB media and incubated for 6 hours at 37°C shaker with vigorous shaking. Then 25 mL of the cell culture were aseptically transferred to a sterile, ice-cold 50 mL disposable polypropylene tubes and stored on ice for 10 min. The cells were recovered by centrifugation at 4000 rpm for 10 min at 4°C. The pellet was resuspended in 5 mL of ice-cold 0.1 M $CaCl_2$ and stood on ice for 10 min. The cells were centrifuged again, and the tubes were drained thoroughly. The pellet was resuspended in 1 mL of ice-cold 0.1 M $CaCl_2$.

7.2. Transformation of competent cells

100 μL of competent cell suspension from the above procedure were transferred to a sterile microfuge tube using a chilled, sterile pipette tip. 500 ng PCR mutated plasmid DNA was added to the tube. It was placed in a 42°C water bath for exactly 90 sec. Then the tube was rapidly transferred to an ice bath for 1 min. LB (400 μL) containing 60 μg/mL ampicillin was added to the tube and the tube was incubated for 45 min in 37°C water bath. 200 μL transformed competent cells were spreaded onto the LB-ampicillin agar plate.

8. Purification of threonine dehydrogenase from extracts of *E.coli* SP1192 pTDH E88C

8.1. Growth of *E.coli* SP1192 pTDH E88C cells.

One colony from the above LB-ampicillin agar plate was picked up sterilely, then cultured and harvested as described previously in p20.

8.2. Preparation of the crude extract from *E.coli* SP1192 pTDH E88 cells

E.coli cells were resuspended in cold 0.05 M tris-HCl, pH 8.4 (0.5 mM 2-mercaptoethanol and 0.02% NaN_3 were added to all buffers). Two mL of buffer was used for each gram of cells. The cell membranes were fractured by sonic oscillation in the Branson sonicator in 2 min burst for each 5 g of cells (the cells were kept in an ice bath to maintain the temperature at or below 10°C). The resulting homogenate was centrifuged at 20,000 xg for 30 min. The supernatant was filtered by Acrodisc PF 0.8/0.2 μm two-stage filter and stored at 4°C. A 0.5 mL aliquot was saved in the –20°C freezer for later analysis.

8.3. First step of purification : gel filtration chromatography

A gel filtration column was prepared by gravity packing TOYOPEARL® HW-55s media into a Waters AP-2 (2 cm ID x 60 cm) column, which was connected to a Water 650E Protein Purification System. Crude extract (5 mL) was injected for each run. The buffer was 20 mM tris

pH 8.5 containing 0.1 M KCl. The flow rate was set at 1 mL/min. Threonine dehydrogenase activity was determined by colorimertric microplate assay. Active fractions were pooled together and a 0.5 mL aliquot was saved at –20°C freezer for analysis.

8.4. Second step of purification: dye-ligand affinity chromatography

A dye-ligand affinity chromatography column was prepared by gravity packing Cibacron Blue F3GA (Reactive Blue 2 Sepharose) into a Waters AP-2 (1cm ID X 10 cm) column which was connected to Water 650E Protein Purification System. The flow rate was maintained at 2 mL/min. The gel filtration pool (1 mL) was applied to the column at pH 7.5. Then the column was washed with Tris buffer (pH 7.5) until the absorbance (λ=280 nm) returned to baseline. In order to elute TDH activity, 5 mL pH 8.5 0.02 M Tris buffer containing 5mM NAD^+ was washed through the column. TDH activity was assayed by colorimertric microplate assay and active fractions were pooled together. A 0.5 mL aliquot was saved at –20°C freezer for later analysis.

8.5. Third step of purification: ion exchange chromatography

A Poros 50 HQ (10 mm ID X 50 mm) High Capacity Strong Anion Exchange Column was used for this last step of purification. Before applying the sample to the column, the fraction pool from Cibacron Blue F3GA was concentrated using AMICON CENTRIPLUS® Concentrators and centrifuged at 3000 x g for 125 min. The concentrated sample was applied to the Poros HQ column at 1 ml volume each run.

The program gradient is shown below:

- Reservoir A is a 100 mM solution of the acid form of the buffer (e.g., Tris-HCl)
- Reservoir B is a 100 mM solution of the base form of the buffer (e.g., Tris).
- Reservoir C is 2 M KCl
- Reservoir D is nanopure water.

Time (min)	Flow (mL/min)	%A	%B	%C	%D
initial	4	6	14	0	80
5	4	6	14	0	80
6	4	6	14	5	75
7	4	6	14	5	75
12	4	6	14	10	70
13	4	6	14	10	70
18	4	6	14	15	65
20	4	6	14	15	65
25	4	6	14	20	65
26	4	6	14	20	65
30	4	6	14	80	0
40	0	6	14	80	0

9. Determination of enzyme homogeneity by SDS-PAGE

Polyacrylamide gel electrophoresis was carried out to determine the homogeneity of samples saved from each purification step. The samples were diluted with an equal volume of 2x Laemmli (1 mL 0.5 M Tris-HCl (pH 8.8) solution: 4 mL distilled water, 0.8mLglycerol, 1.6mL 10% SDS, 0.4 mL 2-mercaptoethanol and 0.2 mL of 0.5% bromophenol blue). The gel consisted of a 10% acrylamide separating gel and a 4% stacking gel. Electrophoresis was carried out in Tris·glycine·SDS buffer (0.3% Tris base, 1.45% glycine, 0.1% SDS, pH 8.0) at 200 volts for about 40 min until the tracking dye reached the bottom of the gel. The gel was stained in Coomassie Blue stain solution which consisted of 40% methanol, 10% acetic acid, 50% ddH_2O and 0.1% (w/v) Coomassie Blue for 1 hour, then destained in 40% methanol, 10% acetic acid and 50% water for several hours until Coomassie blue stain was gone. Relative migration (R_m) was determined by dividing the migration distance of the protein bands by that of the tracking dye.

10. Protein determination

Protein concentration in solution was determined using the Bio-Rad version of the Bradford dye-binding assay.[66] Bovine gamma globulin served as standard and a calibration curve was prepared. The Dye Reagent Concentrate was diluted 1:4 with ddH_2O and filtered through a

Whatman #1 filter to remove particles. Crude extract and aliquot samples saved from each purification step were mixed with water to a total volume of 150 μL in all tubes (the dilution and volume of each sample was ajustified to give the absorbance reading falling in the range of the calibration graph). Then 5 mL of diluted dye reagent was added to each tube and vortexed well. The mixtures were incubated at room temperature for 5 min. Absorbance values at 595 nm were measured for all the samples using a disposable cuvette by a UV-Visible spectrophotometer. The concentration of the TDH for each sample was determined based on the calibration curve.

11. Specific assays for threonine dehydrogenase activity

Threonine dehydrogenase activity was assayed by one of two methods. A colorimetric microplate assay was done routinely to detect TDH activity throughout the purification process.[67] The assay solution consisted of 0.2 M Tris-HCl (pH 8.4), 0.125 M Threonine, 5 mM NAD^+, 5 μg/mL phMS, 200 μg/mL pITV. 150 μL assay solution was added to wells in a microtiter plate that contained 50 μL of sample. Then a microplate reader followed the ΔA_{490} for 6 minute. Activity was also detected by visual inspection of the pink color produced in the presence of threonine dehydrogenase activity.

The second assay followed the formation of NADH at 340 nm due to the enzymatic oxidation of threonine. This assay was used in studies with aliquot samples saved after each purification step and crude extract to determine the specific and total activity of the enzyme. The assay solution consisted of 0.2 M Tris-HCl (pH 8.4), 0.125 M Threonine and 5 mM NAD^+. Then Assay solution (1 mL) was added to each of the disposable polystyrene cuvettes containing enzyme solution (10-50 μL) and linear reaction rates were observed for the first 5-6 minutes at 37°C. A series of enzyme dilutions was assayed to ensure that the rate of the reaction is

proportional to enzyme concentration. A molar extinction coefficient of 6.22×10^3 was used for NADH.[68]

RESULTS

1. Site-directed mutagenesis of threonine dehydrogenase in *E.coli* using PCR

Site directed mutagenesis is a process whereby a single amino acid substitution can be made in the sequence of an enzyme by changing the sequence of the gene that codes for that amino acid without modifying the remainder of the primary sequence of the enzyme. It is a powerful technique in better understanding the role of the original amino acid in the enzyme's function and structure.

Our overall goal is to determine the possible role of the glutamate 88 in the reaction catalyzed by threonine dehydrogenase in *E.coli*. Therefore, a mutant E88C (replacement of glutamate 88 with cysteine) was constructed by site directed mutagenesis using PCR technology. The two primers containing the single point mutation are shown on Figure 9. The QuikChange™ Site-Directed Mutagaenesis Kit was used to generate the mutagenized TDH plasmids.

The mutagenesis reaction consists of the plasmid containing the native tdh gene, two primers with a single point mutation of glutamate 88 to cysteine, *pfu* polymerase, dNTPs and reaction buffer containing Mg^{2+}. The mutagenized plasmids were used to transform the supplied Epicurian coli XL1-Blue Supercompetent Cells and streaked on LB-ampicillin plates.

The pWhitescript control plasmid was used to test the efficiency of mutant plasmid generation. It is done by using LB-Amp medium that has been treated with IPTG (an inducer of β-galactosidase expression) and X-gal (a chromogenic substrate for β-galactosidase). Colonies expressing a functional β-galactosidase will appear blue on plates coated with IPTG/X-gal. Colonies without the functional enzyme will be white on the same plates. The pWhitescript

control plasmid is gal$^-$ (β-galactosidase gene is defective). Epicurian coli XL 1-Blue supercompetent cells transformed with this control plasmid appear white on plates, because β-galactosidase activity is not present. The oligonucleotide control primers create a point mutation that reproduces the β-galactosidase gene in the template during PCR amplification. Colonies transformed with the mutated gal$^+$ plasmid are blue phenotype.

Our result showed that greater than 80% of the control colonies contained the mutation and appeared as blue colonies on LB-ampicillin agar plates, containing IPTG and X-gal. The sample reaction that contained 0.2 μg/mL of template, 2.5 μg/mL of each of the two primers (refer to p. 23 for the contents of other components in the reaction) resulted in the growth of about 200 colonies on LB-ampicillin plates. In similar reactions that used 0.1 μg/mL or 1 μg/mL of template (the primers concentration were kept constant) no growth was seen on the LB-ampicillin plates.

Figure 9. Linear version of pTDH and sequences of two mutagenic primers. The bold letters show the codon for glutamate-88 on TDH, bold italic letters show the point mutation coding for cysteine in the mutagenic primers.

2. Agarose gel electrophoresis of DNA

Agarose gel electrophoresis (0.8% agarose) showed the existence of DNA at various stage of the experiment (Figure 10). There were impurities on the lanes containing wild type (lane 3) and mutagenized plasmid TDH (lane 5), but none in the PCR product (lane 4). Traditionally, PCR has been used to create an exponential amplification of a target DNA segment, with all the products of one cycle acting as template for the next cycle. In the QuikChange™ Site-Directed Mutagaenesis Kit, the concentration of the mutagenized plasmid was increased linearly instead of exponentially during PCR because the design of the two primers (Fig. 9) allowed the replication of only the parental strands to take place in each successive round. Therefore, the mutagenized plasmid products were present at low level even after PCR (Figure 10, lane 4). To show the weak PCR band, the gel was taken under overexposed condition.

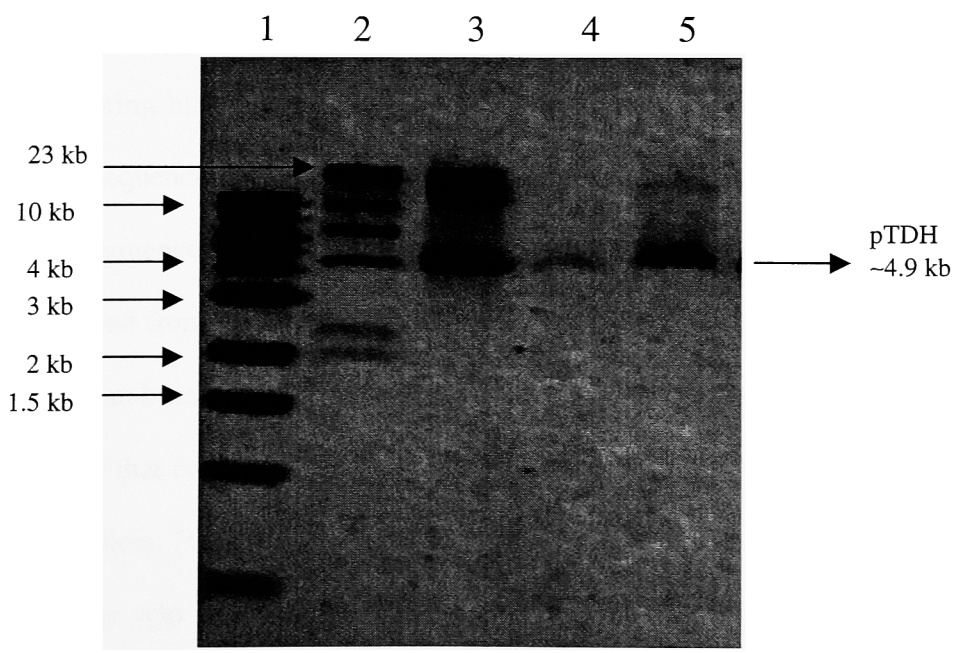

Figure 10. Agrose gel eletrophoresis analysis of various DNA sample. Samples of interest were: lane 1: 1 kb ladder DNA standard; lane 2, λ/HindIII standard; lane 3: plasmid pAJWT; lane 4: PCR mutagenized plasmid TDH; lane 5: mutagenized plasmid isolated from *E.coli* SP1192 after transformation.

3. Cycle sequencing of tdh gene to evaluate the PCR mutagenized plasmid

The positions of the four sequencing primers where it annealed to the template plasmid during PCR are shown on Fig. 8. The sequencing data from University of Rochester Nucleic Acid Laboratory were submitted to BLAST at National Center for Biotechnology information[69] for best matching sequences. The sequencing results with each of the primers are shown in Fig. 11, 12, 13 and 14.

BLAST (Basic Local Alignment Search Tool) is a set of similarity search programs designed to explore all of the available sequence databases regardless of whether the query is protein or DNA. The paragraph before the actual sequence alignment gives the information about the high-scoring segment pairs (HSPs) and some statistical significance between the submitted sequence and database sequence (Expect value is a parameter that tells the number of times one might Expect to see a match simply by chance, P value is the probability of observing a match, ranging from 0-1.[69] When the Expect value is increased from the default value of 10, a larger list with more low-scoring hits can be reported). `Query` refers to the sequence submitted and `Sbjct` refers to database sequence. Vertical lines mean the exact match between the submitted sequence and the database sequence; no line means no match. The letter N means ambiguity in the query sequence, as obtained from the University of Rochester Nucleic Acid Laboratory.

E.coli tdh database sequence is numbered 1704 to 2729. Primer 031 read about 550 bases from 1704 to 2254 that contained the point mutation Glu-88 (GAA) to Cys (TGC). There were ambiguities at positions 2033 and 2199 that were confirmed to be G and G respectively by using primer 636. Primer 636 read sequence from 1939 to 2333 (394 bases length) which also contained the point mutation without any ambiguity. Primer 103 read 399 bases from 2066 to

2465 with one ambiguity 2150 that was confirmed to be the right base (C) by primer 636. Primer 030 read 290 bases from sequence 2439 to 2729 (where tdh gene ends) without any mismatch.

In summary, by using four primers the whole tdh gene of the mutant plasmid were sequenced, the tdh gene was intact with only the expected single point mutation Glu-88 (TGC) to Cys (GAA). To confirm these results DNA sequencing was repeated using plasmid isolated from transformed *E.coli* SP1192 (tdh$^-$).

Figure 11. Sequence data from tdh 1704-2254 using primer 031

emb|X06690|ECKBLTDH *E. coli* genes tdh and kbl
Length = 3563
Plus Strand HSPs:
Score = 2326 (642.7 bits), Expect = 1.3e-215, Sum P(2) = 1.3e-215
Identities = 468/472 (99%), Positives = 468/472 (99%), Strand = Plus / Plus

```
Query:    36 TGAGATGAAAGCGTTATCCAAACTGAAAGCGGAAGAGGGCATCTGGATGACCGACGTTCC 95
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1700 TGAGATGAAAGCGTTATCCAAACTGAAAGCGGAAGAGGGCATCTGGATGACCGACGTTCC 1759

Query:    96 TGTACCGGAACTCGGGCATAACGATCTGCTGATTAAAATCCGTAAAACAGCCATCTGCGG 155
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1760 TGTACCGGAACTCGGGCATAACGATCTGCTGATTAAAATCCGTAAAACAGCCATCTGCGG 1819

Query:   156 GACTGACGTTCACATCTATAACTGGGATGAGTGGTCGCAAAAAACCATCCCGGTGCCGAT 215
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1820 GACTGACGTTCACATCTATAACTGGGATGAGTGGTCGCAAAAAACCATCCCGGTGCCGAT 1879

Query:   216 GGTCGTGGGCCATGAATATGTCGGTGAAGTGGTAGGTATTGGTCAGGAAGTGAAAGGCTT 275
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1880 GGTCGTGGGCCATGAATATGTCGGTGAAGTGGTAGGTATTGGTCAGGAAGTGAAAGGCTT 1939

Query:   276 CAAGATCGGCGATCGCGTTTCTGGCTGCGGCCATATCACCTGTGGTCATTGCCGCAACTG 335
             |||||||||||||||||||||||  ||||||||||||||||||||||||||||||||||
Sbjct:  1940 CAAGATCGGCGATCGCGTTTCTGGCGAAGGCCATATCACCTGTGGTCATTGCCGCAACTG 1999

Query:   336 TCGTGGTGGTCGTACCCATTTGTGCCGCAACACNATAGGCGTTGGTGTTAATCGCCCGGG 395
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Sbjct:  2000 TCGTGGTGGTCGTACCCATTTGTGCCGCAACACGATAGGCGTTGGTGTTAATCGCCCGGG 2059

Query:   396 CTGCTTTGCCGAATATCTGGTGATCCCGGCATTCAACGCCTTCAAAATCCCCGACAATAT 455
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Sbjct:  2060 CTGCTTTGCCGAATATCTGGTGATCCCGGCATTCAACGCCTTCAAAATCCCCGACAATAT 2119

Query:   456 TTCCGATGACTTAGCCGCAATTTTTGATCCCTTCGGTAACGCCGTGCATACC 507
             ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  2120 TTCCGATGACTTAGCCGCAATTTTTGATCCCTTCGGTAACGCCGTGCATACC 2171
```

Score = 418 (115.5 bits), Expect = 1.3e-215, Sum P(2) = 1.3e-215
Identities = 84/85 (98%), Positives = 84/85 (98%), Strand = Plus/Plus

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Query:   507 CCGCGCTGTCGTTTGATCTGGTGGGCGAANATGTGCTGGTTTCTGGTGCAGGCCCGATTG 566
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Sbjct:  2170 CCGCGCTGTCGTTTGATCTGGTGGGCGAAGATGTGCTGGTTTCTGGTGCAGGCCCGATTG 2229

Query:   567 GTATTATGGCAGCGGCGGTGGCGAA 591
             |||||||||||||||||||||||||
Sbjct:  2230 GTATTATGGCAGCGGCGGTGGCGAA 2254
```

Figure 12. Sequence data from tdh 1939-2333 using primer 636

emb|X06690|ECKBLTDH E. coli genes tdh and kbl
Length = 3563
Plus Strand HSPs:
Score = 1948 (538.3 bits), Expect = 9.4e-203, Sum P(3) = 9.4e-203
Identities = 392/395 (99%), Positives = 392/395 (99%), Strand = Plus / Plus

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Query:    30 TCAAGATCGGCGATCGCGTTTCTGGCTGCGGCCATATCACCTGTGGTCATTGCCGCAACT 89
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Sbjct:  1939 TCAAGATCGGCGATCGCGTTTCTGGCGAAGGCCATATCACCTGTGGTCATTGCCGCAACT 1998

Query:    90 GTCGTGGTGGTCGTACCCATTTGTGCCGCAACACGATAGGCGTTGGTGTTAATCGCCCGG 149
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1999 GTCGTGGTGGTCGTACCCATTTGTGCCGCAACACGATAGGCGTTGGTGTTAATCGCCCGG 2058

Query:   150 GCTGCTTTGCCGAATATCTGGTGATCCCGGCATTCAACGCCTTCAAAATCCCCGACAATA 209
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Sbjct:  2059 GCTGCTTTGCCGAATATCTGGTGATCCCGGCATTCAACGCCTTCAAAATCCCCGACAATA 2118

Query:   210 TTTCCGATGACTTAGCCGCAATTTTTGATCCCTTCGGTAACGCCGTGCATACCGCGCTGT 269
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  2119 TTTCCGATGACTTAGCCGCAATTTTTGATCCCTTCGGTAACGCCGTGCATACCGCGCTGT 2178

Query:   270 CGTTTGATCTGGTGGGCGAAGATGTGCTGGTTTCTGGTGCAGGCCCGATTGGTATTATGG 329
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  2179 CGTTTGATCTGGTGGGCGAAGATGTGCTGGTTTCTGGTGCAGGCCCGATTGGTATTATGG 2238


Query:   330 CAGCGGCGGTGGCGAAACACGTTGGTGCACGCAATGTGGTGATCACTGATGTTAACGAAT 389
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  2239 CAGCGGCGGTGGCGAAACACGTTGGTGCACGCAATGTGGTGATCACTGATGTTAACGAAT 2298

Query:   390 ACCGCCTTGAGCTGGCGCGTAAAATGGGTATCACC 424
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Sbjct:  2299 ACCGCCTTGAGCTGGCGCGTAAAATGGGTATCACC 2333
```

Figure 13. Sequence data from tdh 2066-2465 using primer 103

emb|X06690|ECKBLTDH E. coli genes tdh and kbl
Length = 3563
Minus Strand HSPs:
Score = 1993 (550.7 bits), Expect = 4.4e-158, P = 4.4e-158
Identities = 399/400 (99%), Positives = 399/400 (99%), Strand = Minus / Plus

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Query:   400 TGCCGAATATCTGGTGATCCCGGCATTCAACGCCTTCAAAATCCCCGACAATATTTCCGA 341
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Query:   340 TGACTTAGCCGCAATTTTTGATCCNTTCGGTAACGCCGTGCATACCGCGCTGTCGTTTGA 281
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Sbjct:  2126 TGACTTAGCCGCAATTTTTGATCCCTTCGGTAACGCCGTGCATACCGCGCTGTCGTTTGA 2185

Query:   280 TCTGGTGGGCGAAGATGTGCTGGTTTCTGGTGCAGGCCCGATTGGTATTATGGCAGCGGC 221
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Sbjct:  2186 TCTGGTGGGCGAAGATGTGCTGGTTTCTGGTGCAGGCCCGATTGGTATTATGGCAGCGGC 2245

Query:   220 GGTGGCGAAACACGTTGGTGCACGCAATGTGGTGATCACTGATGTTAACGAATACCGCCT 161
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Sbjct:  2246 GGTGGCGAAACACGTTGGTGCACGCAATGTGGTGATCACTGATGTTAACGAATACCGCCT 2305

Query:   160 TGAGCTGGCGCGTAAAATGGGTATCACCCGTGCGGTTAACGTCGCCAAAGAAAATCTCAA 101
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Sbjct:  2306 TGAGCTGGCGCGTAAAATGGGTATCACCCGTGCGGTTAACGTCGCCAAAGAAAATCTCAA 2365

Query:   100 TGACGTGATGGCGGAGTTAGGCATGACCGAAGGTTTTGATGTCGGTCTGGAAATGTCCGG 41
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Sbjct:  2366 TGACGTGATGGCGGAGTTAGGCATGACCGAAGGTTTTGATGTCGGTCTGGAAATGTCCGG 2425

Query:    40 TGCGCCGCCAGCGTTTCGTACCATGCTTGACACCATGAAT 1
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Sbjct:  2426 TGCGCCGCCAGCGTTTCGTACCATGCTTGACACCATGAAT 2465
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Figure 14. Sequence data from tdh 2439-2729 using primer 030

emb|X06690|ECKBLTDH E. coli genes tdh and kbl
Length = 3563
Minus Strand HSPs:
Score = 1610 (444.9 bits), Expect = 4.9e-175, Sum P(6) = 4.9e-175
Identities = 322/322 (100%), Positives = 322/322 (100%), Strand = Minus / Plus

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Query:   400 TTTCGTACCATGCTTGACACCATGAATCACGGCGGCCGTATTGCGATGCTGGGTATTCCG 341
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Sbjct:  2439 TTTCGTACCATGCTTGACACCATGAATCACGGCGGCCGTATTGCGATGCTGGGTATTCCG 2498

Query:   340 CCGTCTGATATGTCTATCGACTGGACCAAAGTGATCTTTAAAGGCTTGTTCATTAAAGGT 281
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Sbjct:  2499 CCGTCTGATATGTCTATCGACTGGACCAAAGTGATCTTTAAAGGCTTGTTCATTAAAGGT 2558

Query:   280 ATTTACGGTCGTGAGATGTTTGAAACCTGGTACAAGATGGCGGCGCTGATTCAGTCTGGC 221
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Sbjct:  2559 ATTTACGGTCGTGAGATGTTTGAAACCTGGTACAAGATGGCGGCGCTGATTCAGTCTGGC 2618

Query:   220 CTCGATCTTTCGCCGATCATTACCCATCGTTTCTCTATCGATGATTTCCAGAAGGGCTTT 161
             |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  2619 CTCGATCTTTCGCCGATCATTACCCATCGTTTCTCTATCGATGATTTCCAGAAGGGCTTT 2678

Query:   160 GACGCTATGCGTTCGGGCCAGTCCGGGAAAGTTATTCTGAGCTGGGATTAACACGAACAA 101
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  2679 GACGCTATGCGTTCGGGCCAGTCCGGGAAAGTTATTCTGAGCTGGGATTAACACGAACAA 2738

Query:   100 GGGCTGGTATTCCAGCCCTTTT 79
             |||||||||||||||||||||
Sbjct:  2739 GGGCTGGTATTCCAGCCCTTTT 2760
```

4. Purification of TDH E88C from extracts of *E.coli* SP1192

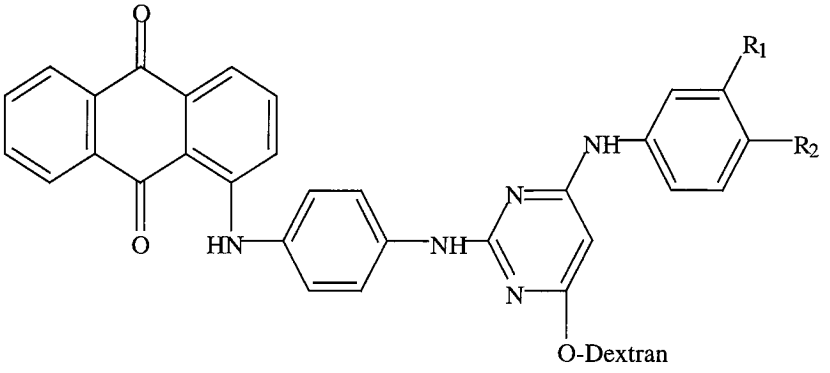
Wild type threonine dehydrogenase was reproducibly obtained in homogeneous form from extracts of *E.coli* SBD76 cells using two chromatography steps-DEAE-Sephadex and Blue Dextran-Sepharose 4B.[52] The second step using blue Dextran-Sepharose 4B was especially effective since the dye (Cibacron Blue F3GA, Fig. 15) was thought to bind specifically to the NAD^+-binding site on the threonine dehydrogenase. All the other proteins in the DEAE ion-exchange pool were washed through the column, leaving only the TDH bound. The bound TDH was then released with an NAD^+ wash.

Attempts were made to purify TDH E88C by exactly the same procedure. However, for the mutated TDH, there was always one other protein band that coeluted with the TDH when the blue column was washed with buffer containing NAD^+. This was still a crucial step during purification, since it removed most of the other proteins present after the ion exchange step. Therefore, a modified procedure was developed. The crude extracts containing TDH E88C was applied to TOYOPEAL® HW-55S gel filtration column (elution profile for TDH activity is shown on Figure 16). Active fractions were pooled and applied to a Cibacron Blue F3GA dye-ligand affinity column (elution profile for TDH activity is shown on Figure 17), The active fraction pools were concentrated and finally applied to a HQ ion-exchange column (elution profile for TDH activity is shown on Figure 18).

SDS-PAGE gel electrophoresis showed a single band after the HQ ion-exchange column, which demonstrated that TDH E88C was obtained in homogenous form after three chromatography steps (Figure 19). Based on the molecular weight standard curve (log MW vs. R_m, see Figure 20), the subunit molecular weight of purified TDH band is about 38,800, so the estimated molecular weight of TDH in its tetrameric form is 155,000, while the literature value is

148,000[51]. The protein determination and TDH activity assay are summarized in table III. As seen from table IV, most of the protein was lost during reactive blue affinity column (protein yield goes from 74% down t o17%), which is consistent with the elution profile (Fig. 17) of the affinity column for mutant TDH that the spectrum has a very wide peak. Specific activity of pure TDH E88C mutant is only 0.177 U/mg, which is decreased dramatically compared to the wild type TDH.

Figure 15. Structure of Cibacron Blue F3GA



R_1 = SO_2ONa or H
R_2 = H or SO_2ONa

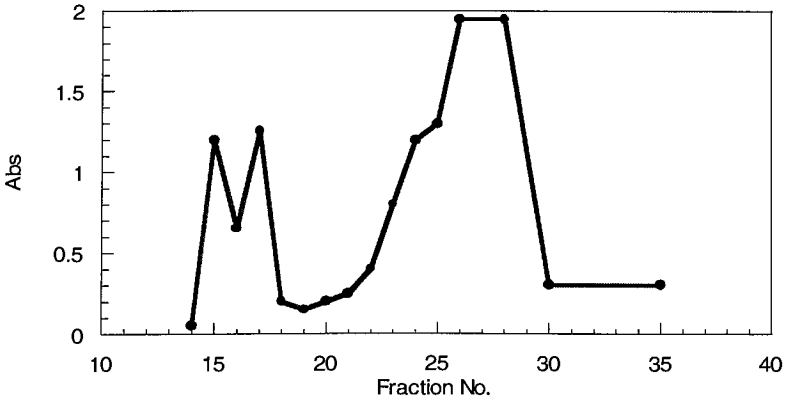


Figure 16. Elution profile for TDH activity for the gel filtration column. Injection volume: 5 mL crude extract. Flow rate: 2 mL/min. Buffer: 20 mM Tris, pH 8.5 with 100 mM KCl. Detector wavelength at 290 nm, AUFS=2.0. Fraction collector: 4 min/tube. TDH activity was detected at fraction 24 and 25.

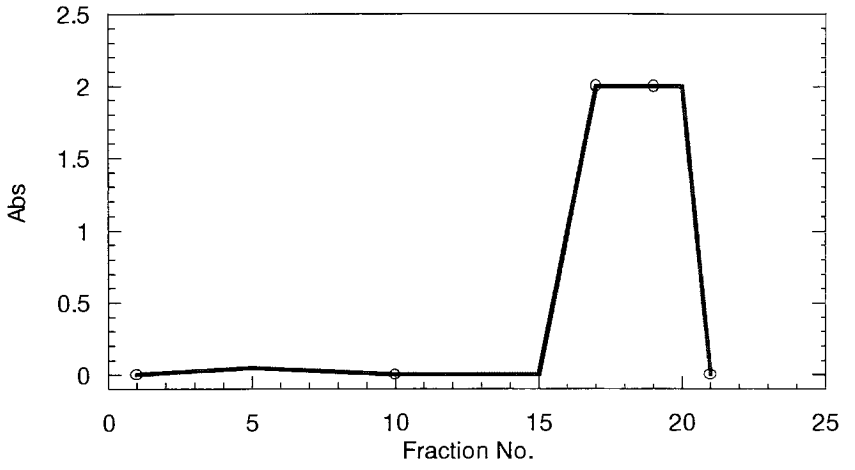


Figure 17. Elution profile for TDH activity for blue dye affinity column. Injection volume: 1 mL. Flow rate: 2 mL/min. Loading buffer: 20 mM Tris, pH 7.5 with 100 mM KCl. Elution buffer: 20 mM Tris pH 8.5 with 100 mM KCl and 5 mM NAD. Detector wavelength at 280 nm, AUFS=2.0. Fraction collector: 1 min/tube. TDH activity was detected at fraction 17 and 18.

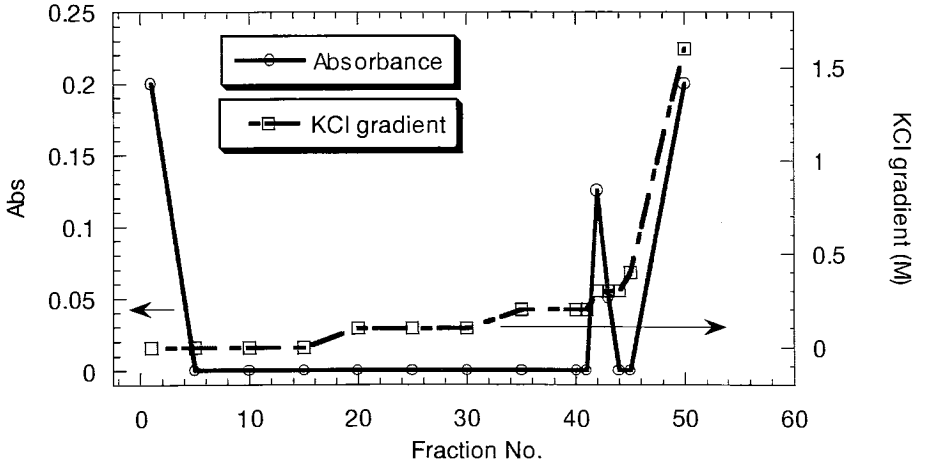


Figure 18. Elution profile for TDH activity from HQ ion-exchange column. Injection volume: 1 mL. Flow rate: 4 mL/min. Buffer: 20 mM Tris pH 8.5 with KCl gradient. Detector wavelength at 280 nm, AUFS=2.0. Fraction collector: 0.5 min/tube. TDH activity was detected at fraction 42 when salt concentration was 300 mM.

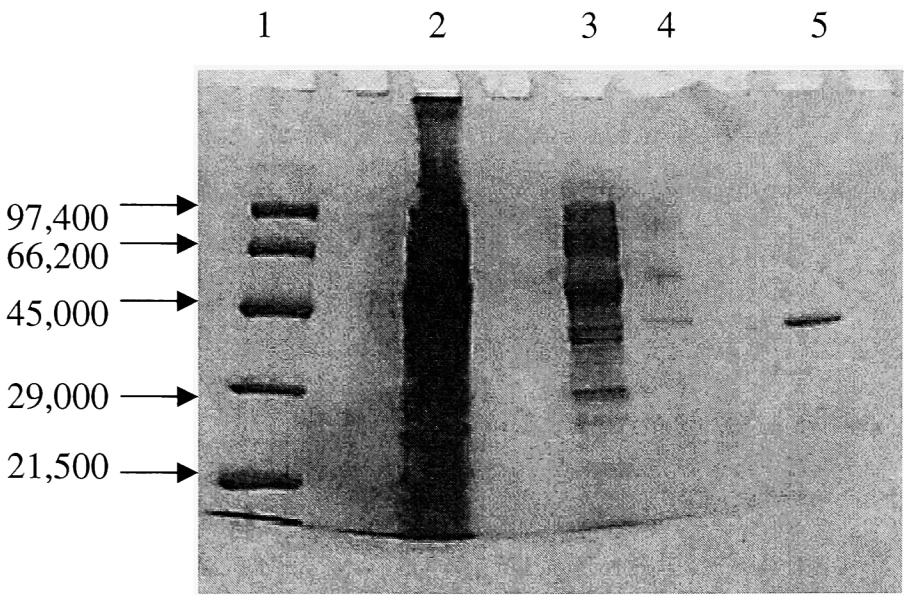


Figure 19. SDS-PAGE Gel Electrophoresis showing samples after each purification step. Lane 1: MW standard; lane 2:crude extract; lane 3: gel filtration; lane 4: blue F3GA; lane 5: HQ ion-exchange.

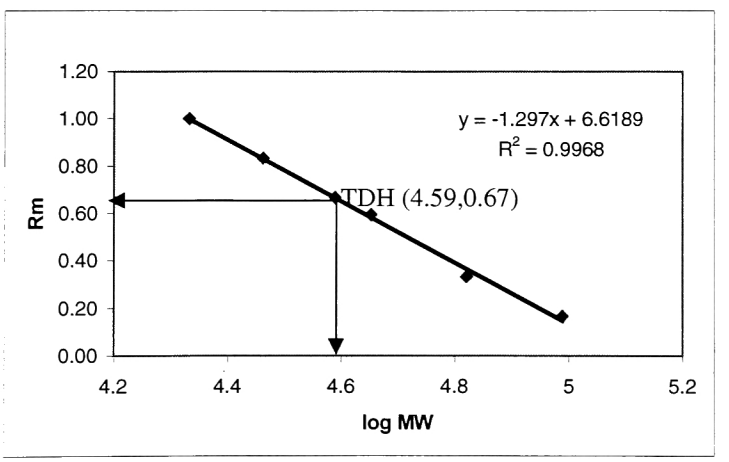


Figure 20. SDS-PAGE standard curve (log MW vs. R_m for molecular weight standard)

Table III. Summary of protein assay and TDH activity assay.

Enzyme Name	Threonine Dehydrogenase			Starting Material: extract of *E.coli* SP1192 pAJ E88C			
Protein Method:	Bradford Protein Assay			Definition of Activity Unit: one unit is defined as the amount of enzyme that catalyzes the formation of 1 umol NADH/mL/min.			
-				Assay Procedure: ΔA_{340}/min			
Fraction	Volume (mL)	Protein (mg/mL)	Total Protein (mg)	Specific Activity (U/mg)	Total Units	Yield (%)	Purification (fold)
Crude Homogenate	5	4.98	24.9	0.067	1.67	100	1
Gel Filtration	8	2.51	20.08	0.062	1.25	74	0.9
Reactive Blue Sepharose	15	0.19	2.85	0.097	0.28	17	1.4
HQ Ion-Exchange	2	0.06	0.12	0.177	0.02	1	2.6

DISCUSSION

❖ **Challenges in generating the mutant enzyme and in purifying the enzyme**

The first challenge encountered in our research was to create the mutated plasmid TDH E88C using PCR. We started with a protocol taken from *Molecular Biology: Current Innovations and Future Trends*.[70] AmpliTaq Gold Taq Polymerase, a chemically modified form of AmpliTaq DNA polymerase from Perkin-Elmer Co. was used to increase the specificity, and sensitivity of PCR product.[71] The polymerase adjunct, Taq Extender PCR additive was added to increase reliability of long sequences.[72] *DpnI* endonuclease (target sequence: 5'-G^{m6}ATC-3') was used to digest parental DNA and select for mutation-containing amplified DNA. *Pfu* DNA polymerase was used to remove any extra bases at the 3' ends of the product added by Taq polymerase. Then the linear PCR product was ligated by T_4 DNA ligase. The protocol seems to have incorporated many techniques, but no band was ever shown on the DNA agarose gel. One possible explanation is that the AmpliTaq Gold Taq Polymerase did not work for us since a few other students tried it without success.

We then moved to the QuikChange Site –Directed Mutagenesis Kit from Stratagene. This kit uses *pfu* DNA polymerase, omitting Taq polymerase entirely. After trying different template concentrations: 5 ng, 10 ng, 20 ng and 50 ng, the right PCR conditions were finally found (DNA template concentration at 10 ng) as described in p. 23 and the mutant was obtained. PCR is a very simple, widely used reliable technique, but establishing a good condition for each individual experiment can be time-consuming and tricky. Good lab practice is especially important when dealing with the microquantities employed in PCR.

Another challenge was to purify the mutant TDH. Our first attempt was to follow the method that reproducibly yielded obtained homogeneous wild type TDH from extracts of *E.coli* SBD76 cells, using two chromatography steps: DEAE-Sephadex followed by Blue Dextran-Sepharose 4B.[52] However, for the mutant TDH, there was always one other protein band that coeluted with the TDH. Therefore, a modified three-step chromotographic procedure was developed. The crude extracts containing TDH E88C was applied to TOYOPEAL® HW-55S gel filtration column, active fractions were pooled and applied to a Cibacron Blue F3GA dye-ligand affinity column, then the active fraction pools were concentrated and finally applied to a HQ ion-exchange column. SDS-PAGE gel electrophoresis showed that TDH E88C was finally obtained in homogenous form.

❖ **Mutations to glutamate or aspartate residues in ADH**

Table IV listed some of the mutations that have been done on alcohol dehydrogenases. Below is a more detailed description on two studies that reveal the function of carboxylate groups near the active zinc site.

A Glu residue in the sorbital dehydrogenase has been postulated to replace the cysteine of alcohol dehydrogenase as a ligand to the active-site zinc atom based on modeling the primary structure of sheep liver SDH[13] to the three-dimensional structure of horse liver alcohol dehydrogenate and extended X-ray absorption fine-structure studies.[73] Site-directed mutagenesis was carried out on rat SDH[74] to replace Glu155 with a Cys to mimic the alcohol dehydrogenase relationships, and an Ala. Glu155Ala creates an inactive enzyme, showing that proper active-site interaction is lost. Cys substitution is active, but the catalytic efficiency decreases markedly. The results strongly suggest that Glu155 is a ligand to the active-site zinc atom in mammalian sorbitol dehydrogenase.[75]

Another study was done on yeast alcohol dehydrogenase[76] to replace Glu68 to glutamine and Asp49 to Asparagine (residue numbering as for horse liver enzyme). The results show that removing the negative charges near the active site zinc considerably decreases catalysis. Gln substitution at position 68 reduced the catalytic efficiency by 100-fold for both ethanol oxidation and acetaldehyde reduction; Asn substitution at position 49 decreased efficiencies 1000-fold. Turnover numbers with ethanol decreased by about 40-fold and affinities for coenzymes, substrates, and inhibitors decreased by up to 20-fold.[76]

Although the carboxyl groups of Asp49 and Glu68 of yeast alcohol dehydrogenase are not direct ligands to the active site zinc based on the structures of HLADH,[77] they appear necessary to modulate the effective charge on the zinc ion and create an favorable electrostatic environment for rearrangements around the catalytic zinc. The mutations may slightly alter the secondary structure near the active site zinc by the removal of negative charges and result in poor binding of the coenzymes and substrates with the enzyme. Secondly, the increased polarizing electrostatic potential near the active site zinc, due to removal of negative charges, decreases the pK_a value of the alcohol bound to zinc. This causes slower hydride transfer which decreases the activity of mutants for oxidizing ethanol. Therefore, two basic effects-structural perturbations and electrostatic effects should be considered in explaining the altered kinetics of the mutant enzymes.[80]

Table IV List of site directed mutagenesis in alcohol dehydrogenase

Mutation	Effect	Proposed Role
Human class I ADH[78, 79, 80] ($\beta\beta$ isoenzyme) Arg47Lys Arg47His Arg47gln	All the mutants result in enzymes with lower affinity for coenzyme than wild-type $\beta_1\beta_1$.	Agr47 contributes to the tight binding of NAD^+ (NADH) exhibited by $\beta_1\beta_1$.
Phe93Ala/ Thr94Ile Double mutant	The V_{max}/K_m value of mutant for secondary alcohol and stereoselectivity for enantiomeric alcohol are similar to that of $\alpha\alpha$ but the dependency of V_{max}/K_m on primary alcohol chain length is similar to $\beta_1\beta_1$, not $\alpha\alpha$.	Position 93 and 94 account for substrate specificity between isoenzyme $\beta_1\beta_1$ and $\alpha\alpha$ to some extends.
Thr48Ser Thr48Ala	The T48S mutant is inhibited by testosterone and has steroid dehydrogenase activity as $\gamma\gamma$. Meanwhile several kinetic constants are affected in the direction toward those characteristics of the $\gamma\gamma$ isozyme too. Alanine at position 48 creates an inactive enzyme.	Position 48 is necessary to obtain isozyme specificity between $\beta\beta$ and $\gamma\gamma$.
Yeast ADH I[76] Asp49Asn Glu68Gln	The catalytic efficiencies for ethanol oxidation and acetaldehyde reduction are greatly reduced and the affinity of the enzymes for NAD^+ is decreased too.	Position 49 and 68 are in the second sphere of the zinc site but they are involved in catalysis.
Cinnamyl ADH[81] Ser212Asp	The $K_{m\ (NADPH)}$ of the mutant is much higher than the K_m for WT enzyme, while the k_{cat}/K_m ratio with NADPH was decreased 25 fold.	S212 is involved in recognizing the coenzyme NADPH.
Rat SDH[75] Glu155Cys Glu155Ala	E155A has no activity. E155C is partly active, but very unstable.	Glu155 is a ligand to the active-site zinc atom.
Human class IV ADH[82] Phe309Leu/ Cys317Ala double mutant	Mutations at these two residues dramatically increase the K_m values toward small alcohol substrates, but the K_m values for substrates with five or more carbons are less affected.	Substrate binding pocket. The interactions between the enzyme and smaller substrates are affected while the binding of longer alcohol is not.

Mutation	Effect	Proposed roles
Human class III ADH[83, 74, 84] Arg115Ala Arg115Asp	The activities of the mutant enzymes toward ethanol are essentially identical to the native enzyme, but mutagenesis greatly decrease the k_{cat}/K_m for glutathione-dependent formaldehyde oxidation and their activation by fatty acids are markedly attenuated.	Substrate binding pocket for glutathione-dependent formaldehyde dehydrogenase activity.
Cys97Ala Cys97Glu Cys100Ala Cys103Ala Cys111Ala	All mutations result in inactive, unstable enzymes. Northern-blot analysis revealed the presence of the expected mRNAs from expression plasmids constructed with the different mutated and non-mutated ADH, and Western-blot analysis give faint signals for the mutated recombinant proteins from crude extracts.	Cys97, 100, 103 and 111 are the ligands to the structural zinc and the structural role of the zinc atom may involve conservation of interfaces regulating the enzyme quaternary structure.
Asp57Leu Tyr93Phe Asp57Leu/ Tyr93Phe	N57L, Y93F and the double mutant all cause considerable loss of the formaldehyde dehydrogenase activity. At the same time, the alcohol dehydrogenase activity of the double mutant has gained a characteristic class I property.	Position57 and 93 are important for class differentiation. Exchange at these positions from class III wild-type to class I wild-type residues, transforms the enzyme properties.
Drosophila ADH[85, 86, 87] Gly14Val Gly14Ala Cys135Ala Cys218Ala Cys135Ala/ Cys218Ala	G14V is virtually inactive, and G14A causes a 31% decrease in activity. Thermal denaturation and kinetic and inhibition further demonstrate that replacing glycine-14 with either alanine or valine leads to structural changes in the NAD binding domain. All the cysteine mutants cause no decreases in the catalytic activity of the enzyme.	Gly14 plays a crucial role in maintaining the correct conformation in the NAD binding domain. Neither of the cysteine residues is essential for catalysis.
Tyr152Phe Tyr152Glu Tyr152His Tyr152Cys Lys156Ile Lys156Arg	Y152F, Y152Q, Y152H and K156I are catalytically inactive. The activities of Y152C and K156R toward ethanol are greatly decreased, but the $K_{m\ (NAD)}$ is essentially unaltered. Furthermore, these two mutant have different substrate specificity and behave differently on competitive inhibition than wild-type ADH.	Tyr-152 and Lys-156 have essential roles in the interaction of the substrate with the enzyme, but not in the binding of the cofactor to the enzyme.
Gly19Ala Asp38Ala Pro214Ser	Mutations at position 19, 38 and 214 affect the K_m for NAD without altering the K_m for alcohol. Unlike the wild type, G19A shows no activity when the cofactor NADP is used.	Asp38 and Pro214 interact with the cofactor NAD. Gly19 plays a role in the recognition of cofactor NADP and NAD.

❖ **Mutations in *E.coli* TDH**

Random mutagenesis with hydroxylamine was done on TDH from *E.coli* by Yen-Wen Chen.[88] The mutants were divided into 4 groups:

(1) *Amino acid changes that are possible ligands to* Zn^{2+}. Mutant Cys38Tyr shows no TDH activity, which confirms Cys38 is a ligand to the active site zinc. Pro149Ser and Gly62Asp mutants are next to two other possible Zn-ligands (His-63 and Asp-148) and show significant effect on TDH activity.

(2) *Mutations on a few conserved glycine residues.* Glycine residue is important in protein folding because its short side chain can fit unusual conformational changes. Gly62Asp, Gly263Asp and Gly285Asp all result in inactive enzymes. The substitution of Asp for gly disrupts the secondary structure and protein folding.

(3) *Substitutions at the substrate or coenzyme binding site.* Mutations Gly89Asp, Ala172Val, Gly171Asp and Glu238Lys belong to this group. Based on homology to the 3-D structure of LADH, Gly-89 is one of the residues that form the substrate-binding pocket. The other three residues are in the NAD^+ binding site.

(4) *Mutations for further research.* The results of some of the mutations such as Arg97Cys, Arg97His, Gly114Ser, Gly119Ser,Gly263Asp and Glu88Lys can't be explained by the current knowledge, which demands further study.

Site-directed mutagenesis of His90 and Cys38 of *E.coli* TDH was done by Adam R. Johnson.[28] His90 was changed to Arg, Asp and Ala. All three mutants show decreased TDH activity and altered substrate specificity which suggest that His-90 plays a role in the formation substrate binding pocket. Another mutation was Cys38Glu. The mutant retains only about 1% of

the level of enzymatic activity of WT TDH. Therefore, it supports the idea that Cys38 is a ligand to the active site zinc.

Our experiments found that the specific activity of pure TDH E88C mutant was 0.177 U/mg, the total activity for the crude extract is 1.67 U. Pure TDH of *E.coli* K-12, designated SBD76 shows specific activity of 34 U/mg, while the total activity for the crude extract is 2463 U.[89] One unit of enzyme activity is the amount that catalyzed the formation of 1.0 μmol of NADH/min. It is obvious that the total activity and specificity activity of the TDH E88C mutant is significantly decreased compared to the wild type TDH. The loss of the activity of the mutant might be due to many factors such as material loss or the loss of catalytic zinc during three-step chromatography purification. Glu-88 may be involved in electrostatic stabilization of the active site Zn^{2+}, as was seem in Yeast ADH I.[80] No data on steady state kinetics value has been obtained thus far, making it impossible to give a comprehensive explanation of the role of the E88C playing in the enzymatic catalysis.

Further studies should be done in order to fully understand the function of glutamate 88:

- Construction of another mutant, Glu88Ala, using site-directed mutagenesis to see if this is an essential residue.
- Comparison of chromatographic behavior of three enzyme forms.
- Determination of the zinc content of both the wild-type and mutant forms of TDH E88C and E88A using atomic absorption spectrophotometric analyses.
- Circular dichroism spectra of WT TDH, TDH E88C and E88A to detect the secondary structure of each protein.
- Chemical characterization of TDH wild type, TDH E88C and TDH E88A: stability to heat, effect of pH on the activity, assay for total SH groups on each enzyme form, peptide mapping

by HPLC or Capillary Eletrophoresis to see if the mutations change the digestion patterns, etc.

- Steady state enzyme kinetics of TDH E88C, TDH E88A, and TDH WT.

In summary, the mutant TDH E88C has been generated using PCR methods, the sequence of the mutant gene has been confirmed, and the protein has been purified to homogeneity. TDH E88C displays greatly deceased total activity and specific activity compared to TDH WT. Further experiments are necessary to clearly define the role of Glu-88 in the structure and function of TDH.

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